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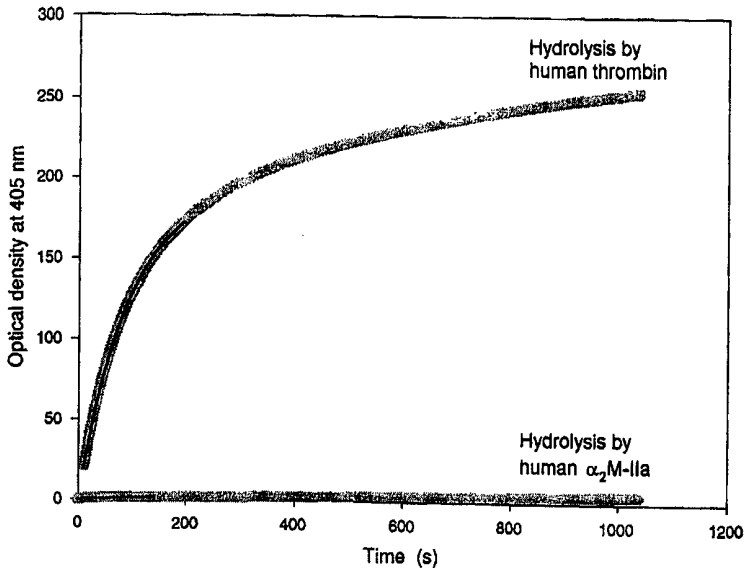
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(57) Abstract

A process is provided for the assessment of an active proteolytic enzyme in a blood or another biological fluid sample possibly comprising a complex of said proteolytic enzyme and α_2–macroglobulin, wherein said sample is contacted with a substrate comprising a molecule of sufficient size coupled to a signal–substrate, said signal–substrate comprising a detectable leaving group, wherein said substrate is hydrolysed by said proteolytic enzyme but not by said complex. The proteolytic enzyme is preferably selected from the group consisting of thrombin, activated clotting factor, activated fibrinolytic factor, and activated component of the complement system. Of these, thrombin is most preferred. The molecules of sufficient size are preferably water soluble and selected from the group consisting of inert protein, preferably ovalbumin, polysaccharide, and synthetic polymer. The size of these molecules is such that they will not fit into the cavity of the α_2M molecules.

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DETERMINATION OF BIOLOGICALLY ACTIVE FORMS OF PROTEOLYTIC ENZYMES

Field of the Invention

The present invention is in the field of diagnostics and relates more in particular to a method for the determination of biologically active forms of proteolytic enzymes, such as thrombin, in blood and other fluids.

Background of the Invention

In the Western world, arterial and venous thrombosis and atherosclerosis together are currently responsible for well over 50% of all mortality and serious morbidity. In a few decades this will be the case world-wide [Murray, C.J. and A.D. Lopez, Science (1996) **274**:740-743]. Thrombosis is caused by an overactivity of the haemostatic mechanism that is responsible for the arrest of bleeding from a wound. This haemostatic-thrombotic system (HTS) is a complex interaction between vessel wall, blood cells, especially blood platelets, and plasma proteins. See, e.g., Hemker H.C. *Thrombin generation, an essential step in haemostasis and thrombosis.* In: Haemostasis and thrombosis, A.L. Bloom *et al.*, eds., Churchill Livingstone, Edinburgh, (1994) 477-490.

In blood there are at least three series of proenzyme-enzyme cascades of great biological importance: the blood coagulation system, the fibrinolytic system and the complement system. In each of these systems proteolytic proenzymes are activated and subsequently inhibited. In general it is important that the concentration of an activated enzyme in such a cascade can be measured in order to assess the function of the system. In the following a typical description of the clotting system will be given with an emphasis on its most important enzyme, thrombin. It is to be noted, however, that the present invention also relates to other clotting enzymes and enzymes of the fibrinolytic and complement pathway.

The mechanism of thrombin generation can be outlined in some detail as follows. In the plasmatic coagulation system factor Xa is formed by the action of the tissue factor/factor VIIa complex (TF-VIIa). Factor Xa binds to tissue factor pathway inhibitor (TFPI) and the TFPI-Xa complex inhibits the TF-VIIa complex. Thrombin activates factors V, VIII and XI and so accelerates its own generation,

but it also binds to thrombomodulin and so starts the protein C mechanism that breaks down factors V and VIII, thus, indirectly, inhibiting further thrombin generation. An important fraction (≈30%) of all thrombin formed in clotting plasma is bound to the fibrin clot. Clot-bound thrombin does retain its thrombotic properties, it can clot fibrinogen, activate factors V, VIII and XI as well as platelets [Béguin, S. and R. Kumar, Thromb. Haemost. (1997) **78**:590-594; Kumar, R., S. Béguin, and H.C. Hemker, Thromb. Haemost. (1994) **72**:713-721, and (1995) **74**:962-968]. It is not inhibited by antithrombin.

Thrombin action causes receptors in the platelet membrane to bind fibrinogen, which causes platelet aggregation. Platelet activation also leads to the exposure of procoagulant phospholipids in a Von Willebrand factor dependent reaction [Béguin S., R. Kumar, I. Keularts, U. Seligsohn, B.C. Coller and H.C. Hemker, *Fibrin-Dependent Platelet Procoagulant Activity Requires GPIb Receptors and Von Willebrand Factor,* Blood (1999) **93**:564-570; Béguin, S. and R. Kumar, *supra* (1997)]. These phospholipids are required for the proper activation of factor X and prothrombin. Recently, the picture has been complicated by the discovery that fibrin, previously thought to be the inert endproduct of coagulation, plays an active role itself. It binds and activates Von Willebrand factor, which activates platelets and provokes the exposure of procoagulant phospholipids via an alternative pathway.

The cooperation between platelets and the coagulation system, including fibrin, is central to the haemostatic-thrombotic system. The mechanism shows an abundance of positive and negative, often nested, feedback loops. Underactivity causes bleeding, overactivity causes thrombosis. Thrombosis manifests itself as coronary infarction, stroke, pulmonary embolism and a large number of less frequent diseases.

In order to assess the function of such a system, also for diagnostic purposes and for the safe use of antithrombotic drugs, a probe is needed for the functional status of the haemostatic-thrombotic system. An important function test of the HTS is the thrombin generation curve (TGC). Carried out in platelet-poor

plasma, it gives information about the function of the plasmatic clotting system. In platelet-rich plasma it measures also the function of the platelets.

According to the prior art the TGC can be measured by subsampling or continuously. In the ancient subsampling method [Biggs, R. and R.G. Macfarlane, *Human Blood Coagulation and its Disorders*. 1953, Oxford: Blackwell; Quick, A.J., *Haemorrhagic Diseases*. 1957, Philadelphia: Lea & Febiger], samples are taken from a clotting mixture, and the concentration of thrombin is measured in each sample.

Active thrombin survives in plasma for only a limited period of time (the half life time is 16-17 s). This is due to circulating antithrombins. Most thrombin (64%) is inactivated by antithrombin (AT), a plasma-protein of 57 kD, 23% by α_2-macroglobulin (α_2M), a 725 kD plasma-protein, and 13% by various other agents [Hemker, H.C., G.M. Willems, and S. Béguin, *A computer assisted method to obtain the prothrombin activation velocity in whole plasma independent of thrombin decay processes,* Thromb. Haemost. (1986) **56**:9-17]. The α_2-macroglobulin-thrombin complex (α_2M-IIa) has the peculiarity that it is inactive towards all macromolecular substrates, but retains its activity against small molecular weight (artificial) substrates. α_2-Macroglobulin is a glycoprotein of Mr 725,000, which is present in plasma in a concentration of 2500 mg/L or 3.5 µM. It is a tetramer of identical subunits of 185 kD. The inactivation reaction of an active proteinase or activated clotting factor with α_2M is a three step process: 1°. Formation of a loose complex, 2°. Hydrolysis of a target peptide in α_2M, causing 3°. a rapid conformational change which physically entraps the enzyme molecule within the α_2M molecule. [See for a review: Travis, J. and G.S. Salvesen, *Human plasma proteinase inhibitors.* Ann. Rev. Biochem. (1983) **52:**655-709].

The group of Hemker then developed a continuous method in which thrombin-catalysed product formation from suitable substrates is monitored directly [Hemker, H.C., *et al.*, *Continuous registration of thrombin generation in plasma, its use for the determination of the thrombin potential.* Thromb. Haemost. (1993) **70**:617-624]. The kinetic constants of the substrate are such that, at the concentration used, the rate of product formation is proportional to the amount of

enzyme present (thrombin or α_2M-IIa). The time-course of enzyme activity in the sample can be estimated as the first derivative of the product-time curve.

A drawback of this method is that part of the thrombin in plasma binds to α_2-macroglobulin. α_2-Macroglobulin is the most abundant non-specific protease inhibitor of blood plasma. It quenches the biological activity of proteases (activated clotting factors, activated fibrinolytic enzymes and activated complement factors) without occupying their active centre, so that there is a residual activity on the usual oligopeptide signal-substrates.

This thrombin/α_2-macroglobulin complex has no known physiological activity but is able to cleave chromogenic substrates. Thus, the product formation observed is the result of the combined activities of free thrombin and α_2-macroglobulin-bound thrombin. The relevant data, i.e. the amount of product formed by free thrombin only, can be extracted from the experimental product formation by a mathematical operation. However, the operation has to be carried out on the whole course of the product generation curve. The product formation therefore needs to be monitored continuously. Although the principle of this continuous method is applicable to all substrates that give a product which can be detected in time, e.g. by fluorescent, electrochemical or NMR signals, its application is currently restricted to the use of chromogenic substrates in an optically clear medium, i.e. defibrinated platelet poor plasma (PPP).

Therefore, there is still a need for an efficient method to measure the amount of (active forms of) proteolytic enzymes, such as thrombin, in blood and other fluids, which obviates the drawbacks of the prior art. The present invention provides such a method.

Summary of the Invention

In accordance with the present invention, a process is provided for the assessment of an active proteolytic enzyme in a blood or another biological fluid sample possibly comprising a complex of said proteolytic enzyme and α_2-macroglobulin, wherein said sample is contacted with a substrate comprising a molecule of sufficient size coupled to a signal-substrate, said signal-substrate comprising a

detectable leaving group, wherein said substrate is hydrolysed by said proteolytic enzyme but not by said complex.

The active proteolytic enzyme is preferably selected from the group consisting of thrombin, activated clotting factor, activated fibrinolytic factor, and activated component of the complement system. Of these, thrombin is most preferred.

The molecules of sufficient size are preferably selected from the group consisting of inert protein, preferably ovalbumin, polysaccharide, and synthetic polymer. Preferably, these inert molecules are water soluble and have a size such that they just do not fit into the cavity of the α_2M molecules. A group of preferred inert molecules has a size of about 40 kD and a solubility of at least 40 mg/ml. Most preferred are inert molecules with a size of about 20 kD and a solubility of at least 50 mg/ml.

These and other aspects of the invention will be further outlined in the description which follows.

Brief Description of the Drawings

Figure 1 represents theoretical curves of hydrolysis products formed in clotting plasma or blood by hydrolysis of a small thrombin substrate and a large thrombin substrate.

Figure 2 represents the curves of hydrolysis products of substrate S2288 coupled with ovalbumin by hydrolysis with α_2M-IIa and thrombin, respectively.

Figure 3 represents the fluorescence patterns of the fluorescent substrate Ala-Arg-AMC coupled with ovalbumin by hydrolysis with free thrombin and thrombin inactivated by α_2M, respectively.

Figure 4 shows the results of the determination of the ETP in plasma with SQ68 and OA-Phe-Pip-Lys-pNA.

Detailed Description of the Invention

The present invention is based on the surprising finding that certain signal-substrates for proteolytic enzymes, notably thrombin substrates, are cleft by thrombin but not by the thrombin/α_2-macroglobulin complex. Thus, these signal-substrates are insensitive to the action of proteolytic enzymes when they are bound to α_2-macroglobulin.

As used herein, the term "signal-substrate" means a substrate that is cleaved by proteolytic enzyme(s) present in the medium, from which a leaving group is split off which is detectable by optical, NMR or other methods. Leaving groups which are optical detectable are, for example, p-nitroanilide and 7-amido-4-methyl-coumarin. p-Nitroanilide absorbs at 405 nm and 7-amido-4-methyl-coumarin is fluorescent (excitation at 390 nm and emission at 460 nm). Examples of NMR-active leaving groups are those containing ^{31}P, ^{13}C, or any other atom which can be detected with NMR or a similar technique. Also H^+ ions can be used as leaving group, which can be detected by measuring changes in the pH.

The present invention has the advantage that the total transient enzymatic activity of the free thrombin in the clotting sample can be assessed from the total amount of substrate that is converted, so that the mathematical procedure can be omitted.

The formation of thrombin in clotting blood or plasma arises from prothrombin by an enzymatic controlled reaction, which is called prothrombinase. Initially this reaction is slow, but the rate increases exponentially until the formed thrombin activates the inactivators of the prothrombinase and the prothrombin is exhausted.

We have found that the formation of thrombin can be fitted into the following mathematical formula:

$$FIIa_t = PT \,.\, (1 - e^{-b*t})c \qquad (i)$$

in which $FIIa_t$ is the formation of thrombin in time, PT is the initial amount of prothrombin, b and c are constants and t is the time. By fitting an actually measured thrombin generation curve with this formula one finds a very good agreement between the fit and the data when b = 0.02 and c = 4.

The formed thrombin will react with the plasmatic inhibitors, from which AT and α_2M are the most important. The reaction constants (decay constants) of these reaction are about 20,000 $(M.s)^{-1}$ and 1,500 $(M.s)^{-1}$, respectively.

If the amount of thrombin is to be measured continuously, a thrombin substrate is added to the reaction mixture. By the action of thrombin a molecule is split off from the substrate which can be measured, for example in a spectrophotometer or a fluorometer.

It has now been found, after extensive research and experimentation, that in contrast to small substrates, when using a relatively bulky thrombin substrate, hydrolysis stops when all thrombin has been inactivated, and the total enzymatic activity of thrombin can be measured as an end-point assay.

Accordingly, in one aspect of the present invention there are provided large size chromogenic and fluorogenic and other signal-substrates, to the extent that they cannot penetrate into the cavity of the α_2M molecule and thus are not hydrolysed by α_2M-IIa. This is achieved by coupling the signal-substrate to an inert bulky carrier molecule which results into a "substrate". The coupling is preferably carried out chemically, in a way which is known to a man skilled in the art.

The exclusion limit of the cavity of α_2M in which an active protease is entrapped is about 10 kD (Barrett, A., *Methods in Enzymol.* (1981) **80**:737-754]. Thus, suitable inert carrier molecules are larger than 10 kD and include, for example, proteins, polysaccharides, synthetic polymers. Preferably, the inert molecules have a good solubility. One preferred group of inert molecules has a size such that the molecules just do not fit into the cavity of the α_2M molecule. Another group of preferred inert molecules has a size of about 40 kD and a solubility of at least 40 mg/ml. Most preferred are inert molecules with a size of about 20 kD and a solubility of at least 50 mg/ml.

Suitable and preferred proteins are those which are commercially available, such as ovalbumin (Mr = 45 kD), bovine serum albumin (Mr = 67 kD), human serum albumin (Mr = 67 kD) and hen-egg white lysozyme (Mr = 14.4 kD).

Suitable polysaccharides have a Mr which may vary from a few kD (so that they do not fit into the α_2M cavity) to more than 500 kD. An example of such a polysaccharide is dextran.

Suitable synthetic polymers include hydrophilic water soluble polymers, with a Mr also varying from a few kD to more than 500 kD, such as polyacrylic acid, polyethylene oxide, polyvinyl chloride, poly(1-vinylpyrrolidone-*co*-acrylic acid), poly(2-hydroxyethyl methacrylate), and the like.

Suitable signal-substrates for the purpose of the present invention are compounds with a leaving group which can be easily detected and which is not split by the protease/α_2-macroglobulin complex. The leaving group preferably gives an optical density signal or a fluorescent signal. Examples of such leaving groups include p-nitroanilide and 7-amino-4-coumarin, respectively.

Suitable substrates for the purpose of the present invention include compounds which are obtained by coupling a signal-substrate of relatively low Mr to a inert molecule of sufficient size. Examples of suitable substrates include ovalbumin coupled to H-D-isoleucyl-L-prolyl-L-arginine-p-nitroanilide.2HCl (also referred to as "OA-S2288"), ovalbumin coupled to H-alanyl-L-arginine-7-amido-4-methyl-coumarin (also referred to as "OA-Ala-Arg-AMC"), and ovalbumin coupled to H-D-phenylalanyl-L-prolyl-L-lysine-p-nitroanilide.2HCl (also referred to as "OA-Phe-Pro-Lys-pNA"). Another example of a suitable substrate is ovalbumin coupled to a signal-substrate releasing one or more hydrogen ions as leaving group.

When such a substrate is used to measure the thrombin generation curve (TGC), the temporary thrombin will hydrolyse a certain amount of substrate, after which no further hydrolysis will take place. The amount of the substrate, as well as the kinetic properties thereof, are preferably so chosen that the amount of thrombin generated in the plasma (or other medium) does not consume the substrate completely. The end-level of conversion product formed can be measured and is directly proportional to the surface under the TGC, which is called the endogenous thrombin potential (ETP).

The substrates can be suitably used to measure the biologically active form of proteolytic enzymes in blood and other fluids containing α_2-macroglobulin.

An important application is the generation and disappearance of thrombin in clotting blood, which is an important function test of the haemostatic-thrombotic system (HTS).

Although the present invention is typically described and exemplified for the assessment of (generated) thrombin in blood and other biological fluids, it will be understood that the method is also suitable for the assessment of other proteolytic enzymes in such biological fluids using possible variations and modifications which are clear to the man skilled in the art. Examples of such other proteolytic enzymes include activated clotting factor, activated fibrinolytic factor, and activated component of the complement system.

The invention is further illustrated by the following Examples which should not be construed as limiting the invention in any respect.

Example 1

Hydrolysis of a thrombin substrate in clotting blood or plasma

To blood or plasma 0.5 mM thrombin substrate is added and blood coagulation starts by calcification and a suitable trigger, in this example typically thromboplastin. Figure 1 shows the theoretical curves of the formation of conversion products in time.

The curves (-●- or -o-) show the hydrolysis of a thrombin substrate in clotting plasma. We assume that thrombin formation occurs to formula (i), see the Detailed Description, with the constants mentioned therein. The formed thrombin will react with both AT and α_2M. The AT concentration is set to 2.5 µM and the α_2M concentration to 3.5 µM. The reaction constant of thrombin inactivation by AT is 20,000 $(M.s)^{-1}$, and that of thrombin inactivation by α_2M is 1,450 $(M.s)^{-1}$. To design the curves numerical methods have been used. We used steps of 1 s. After 1 s an amount of thrombin is formed as indicated by formula (i). From 1 to 2 seconds this amount of thrombin will react with AT and α_2M at a rate determined by the above given constants. After this second some AT-thrombin complex (AT-IIa) and α_2M-IIa is formed. These amounts are subtracted from the present amount of thrombin, but new thrombin is formed in this second according to formula (i). From 2 to 3

seconds the new amount of thrombin will react with AT and α_2M (both corrected for the complexes) while new AT-IIa and α_2M-IIa is formed and thus some thrombin is inactivated. This process is repeated during the time span of 15 min. Thus, we see the build up of AT-IIa and α_2M-IIa during the process and a temporary increase of thrombin, followed by complete inactivation of thrombin by AT and α_2M.

By addition of a small thrombin substrate to this mixture two new reactions are introduced: S is hydrolysed by free thrombin and by α_2M-IIa. When S is a large substrate, it will be hydrolysed by the free thrombin, but not by α_2M-IIa. These reactions will follow Michaelis-Menten kinetics: hydrolysis rate = [Thrombin] . k_{cat} . S / (K_m + S).

The amount of hydrolysis product is calculated with the numerical procedure described above. In case of curve -●-, S is a small thrombin substrate and in case of curve -○-, S is a large thrombin substrate.

The thrombin substrate (S) is hydrolysed by thrombin with a K_m of 500 µM and a k_{cat} of 1 s^{-1}. Also α_2M-IIa hydrolyses S, in case of curve -●- K_m is 500 µM and k_{cat} is 0.5 s^{-1}, and in case of curve -○- K_m is 1 M and k_{cat} is zero (i.e. S is not hydrolysed).

In this Example a model substrate is used with the kinetics given above. In actual practice substrates are available with kinetics which are close to this model. Suitable and preferred examples of such substrates are SQ68, Msc-Val-Arg-pNA, DEMZ-Gly-Arg-pNA, and Petachrome TH/SR.

The above procedure is usually applied in a somewhat modified form in that the process is not monitored continuously but samples are taken every 30 sec and the formed conversion product (indicated by the circles ● or ○) measured.

When using a small thrombin substrate, which procedure was routinely used prior to the present invention, curves were obtained as shown by -●-. In order to determine the amount of conversion product formed by the action of free thrombin, the curve was split into two parts, product formation by free thrombin and α_2M-IIa, respectively. Product formation by free thrombin will stop when all thrombin has been inactivated, but product formation by α_2M-IIa will continue. This explains the ongoing linear product formation at the end of curve -●-. The

collection of data measured had to be split into one set for product formation by free thrombin and one set for product formation by α_2M-IIa. For this procedure a mathematical algorithm has been developed [Hemker, H.C., S. Wielders, H. Kessels and S. Béguin, *Continuous registration of thrombin generation in plasma, its use for the determination of the thrombin potential.* Thromb. Haemost. (1973) **70**:617-624]. The principle is the following. At t=0 no thrombin and no α2M-IIa is present and the signal is set to zero. At t=0.5 min (second measured data point) an increased signal is measured due to S hydrolysis by thrombin and α_2M-IIa. A fraction of this signal (k x α2M-IIa concentration) is due to α_2M-IIa. At t=1 min, the signal is increased due to S hydrolysis by free thrombin by the present amount of α_2M-IIa. This procedure is continued until the last data point is reached. From the theoretically derived curve an the actual observations we know that at the end of the curve all free thrombin has been inactivated and the increase of the signal is completely due to S hydrolysis by α_2M-IIa. By choosing the k at which the last part of the curve becomes minimal (i.e. the formation of product is constant in time) the data set can be split into a signal due to S hydrolysis by free thrombin (-○-) and a signal due to the effect of α_2M-IIa (-▽-).

It is noted that the signal caused by the free thrombin reaches a plateau which remains the same, independent on the method used. When a small thrombin substrate is applied, this ceiling has to be determined with a mathematical algorithm. However, when a large thrombin substrate is used according to the present invention, the plateau can be easily determined by reading the end-level of signal.

Example 2

Hydrolysis of ovalbumin substrate OA-S2288 by α_2M-IIa and thrombin

In this example it is shown that by coupling of ovalbumin to S2288 the substrate loses its ability to be hydrolysed by α_2M-IIa, but not by thrombin. This substrate (OA-S2288) was prepared in the following way. A reaction mixture was prepared containing 1 mM S2288, 50 mM sodium carbonate (pH 10.2), 20 mg/ml ovalbumin and 3.6 mg/ml bis(sulfosuccinimidyl)suberate (BS^3) This mixture was

incubated for 30 min at room temperature, then 50 mM Tris (pH 8.2) was added and the mixture was kept at room temperature for 30 min. The mixture was applied to a Sephadex G25 column (16 x 1.6 cm^3) and eluted with a rate of 3 ml/min. The elution buffer was 50 mM HEPES, 100 mM NaCl (pH 7.35). The first peak that eluted from the column contained the ovalbumin coupled to the S2288 (OA-S2288).

The amidolytic activity of human thrombin and human α_2M-IIa towards OA-S2288 was measured. The reaction mixtures contained 22 µM OA-S2288, 50 nM thrombin or α_2M-IIa and 175 mM NaCl, 50 mM Tris-HCl, 2 mg/ml ovalbumin (pH 7.9).

In Figure 2 the amount of free p-nitroanilide (pNA) formed, expressed as milli optical density at 405 nm, is plotted against the time. The reaction temperature was 37°C. The grey curves are the measured data, whereas the black line is obtained by fitting, assuming that product formation occurs according to Michaelis-Menten kinetics.

Product formation in time can be described as: $E \cdot k_{cat} \cdot S_t / (K_m + S_t)$, in which S_t is the substrate concentration in time (i.e. the substrate concentration originally present (S_0) minus the formed product or split substrate, and E is the enzyme that splits the substrate, either thrombin or α_2M-IIa.

The following constants were found: S_0 = 22 mM, K_m = 1.47 µM and k_{cat} = 0.31 s^{-1} for E is thrombin and 0.002 s^{-1} for E is α_2M-IIa.

Example 3

Hydrolysis of ovalbumin substrate OA-Ala-Arg-AMC by free thrombin and thrombin inactivated by α2M.

This example shows that by coupling of ovalbumin to the fluorescent substrate Ala-Arg-AMC, the substrate will not be split anymore by thrombin entrapped in the cavity of α2M. Figure 3 shows that free thrombin splits off the fluorescent group (AMC), but that thrombin inactivated by α2M cannot.

The coupling of ovalbumin to Ala-Arg-AMC was carried out as follows. To 1 ml of a 1 mg/ml ovalbumin in PBS (= 10 mM potassium phosphate, 150 mM

NaCl, pH 7.4) was added 6 µl of 50 mM BS^3 in 100% dimethylsulfoxide. The mixture was incubated at room temperature for 60 min. and dialysed twice against 2 L PBS (each for 2h). Then 5 µl of 100 mM Ala-Arg-AMC was added and incubated at room temperature for 60 min. Finally, the sample was dialysed as before (twice against 2 L PBS).

Figure 3 shows the results of fluorescence measurements in a Fluoroskan Acsent equipped with a thermostated block, a 390 nm excitation and 460 nm emission filter. The assay was carried out at 25°C in 96 well Microfluor W "U" bottomed micro titre plates (Dynex, London, UK) in 10 mM HEPES, 5 mM $CaCl_2$, 0.02% sodium azide, pH 8.0). In case of -o- the vessel contained 5.5 µM OA-AR-AMC (Ala-Arg-AMC coupled to ovalbumin) and 85 nM human thrombin and in case of -∇- 5.5 µM OA-AR-AMC and 65 µM human α2M-IIa. The black symbols (-●- and -▼-) were obtained by fitting assuming that the substrate is hydrolysed by thrombin according to Michaelis-Menten kinetics. From the fitting the following constants were found for hydrolysis by thrombin: the substrate concentration S_0 = 5.5 µM, K_m = 1.2 µM and k_{cat} = 3.3 s^{-1}. For hydrolysis by α_2M-IIa the already derived S_0 and K_m were found and k_{cat} = 0.008 s^{-1}.

Example 4

Determination of the ETP in plasma with SQ68 and OA-Phe-Pip-Lys-pNA

See Figure 4. Thrombin generation was measured as described earlier [Hemker, H.C., Wielders, S., Kessels, H., Béguin, S. *Continuous Registration of Thrombin Generation in Plasma, its Use for the Determination of the Thrombin Potential.* Thromb. Haemost. (1993) **70**:617-624, and Hemker, H.C. and Béguin S. *Thrombin generation in plasma: its assessment via the endogenous thrombin potential.* Thromb. Haemost. (1995) **74**:134-138].

Formation of pNA in clotting plasma to which was added SQ68 (●) or OA-Phe-Pip-Lys-pNA (▼); Pip is pipecolyl. The pNA formation was monitored by measuring the optical density at 405 nm. The substrate concentration was 0.5 mM in both cases. The data set obtained with SQ68 was analysed and split into pNA

formed by free thrombin (⊗) and pNA formed by α_2M-IIa (∇), using the algorithm described in Example 1.

It can be clearly seen that when using a substrate according to the present invention, experimental data are obtained in a very efficient way which represent directly the same biological activity (i.e. the amount of substrate split by free thrombin) as those which are obtained from a complex mathematical dissection of the curve from a conventional substrate.

It is to be understood that modifications and changes to the preferred embodiment of the invention can be made without departing from the spirit and scope of the present invention.

Claims

1. A process for the assessment of an active proteolytic enzyme in a blood or another biological fluid sample possibly comprising a complex of said proteolytic enzyme and α_2-macroglobulin, characterised in that said sample is contacted with a substrate comprising a molecule of sufficient size coupled to a signal-substrate, said signal-substrate comprising a detectable leaving group, wherein said substrate is hydrolysed by said proteolytic enzyme but not by said complex.

2. The process of claim 1, wherein the active proteolytic enzyme is selected from the group consisting of thrombin, activated clotting factor, activated fibrinolytic factor, and activated component of the complement system.

3. The process of claim 2, wherein the activated proteolytic enzyme is thrombin.

4. The process of any one of claims 1-3, wherein the molecule of sufficient size is selected from the group consisting of inert protein, preferably ovalbumin, polysaccharide, and synthetic polymer.

5. The process of any one of the preceding claims, wherein the molecule of sufficient size is water soluble.

6. The process of any one of the preceding claims, wherein the molecule of sufficient size has a size such that it just does not fit into the cavity of the α2M molecule.

7. The process of any one of claims 1-5, wherein the molecule of sufficient size has a size of about 40 kD and a solubility of at least 40 mg/ml.

8. The process of any one of claims 1-5, wherein the molecule of sufficient size has a size of about 20 kD and a solubility of at least 50 mg/ml.

9. The process of any one of the preceding claims, wherein the leaving group gives an optical density signal or a fluorescent signal.

10. The process of claim 9, wherein the leaving group is p-nitroanilide or 7-amino-4-methyl-coumarin.

11. The process of any one of the preceding claims, wherein the substrate is selected from the group consisting of OA-S2288, OA-Ala-Arg-AMC, and OA-Phe-Pro-Lys-pNA.

12. The process of any one of claims 1-10, wherein the substrate comprises a leaving group of one or more hydrogen ions.

13. The process of any one of the preceding claims, wherein the amount and the kinetic properties of the substrate are selected such that the amount of proteolytic enzyme generated in the sample does not consume the substrate completely.

14. The process of any of the preceding claims, wherein the amount of hydrolysed substrate is directly proportional to the area under the generation curve of the proteolytic enzyme.

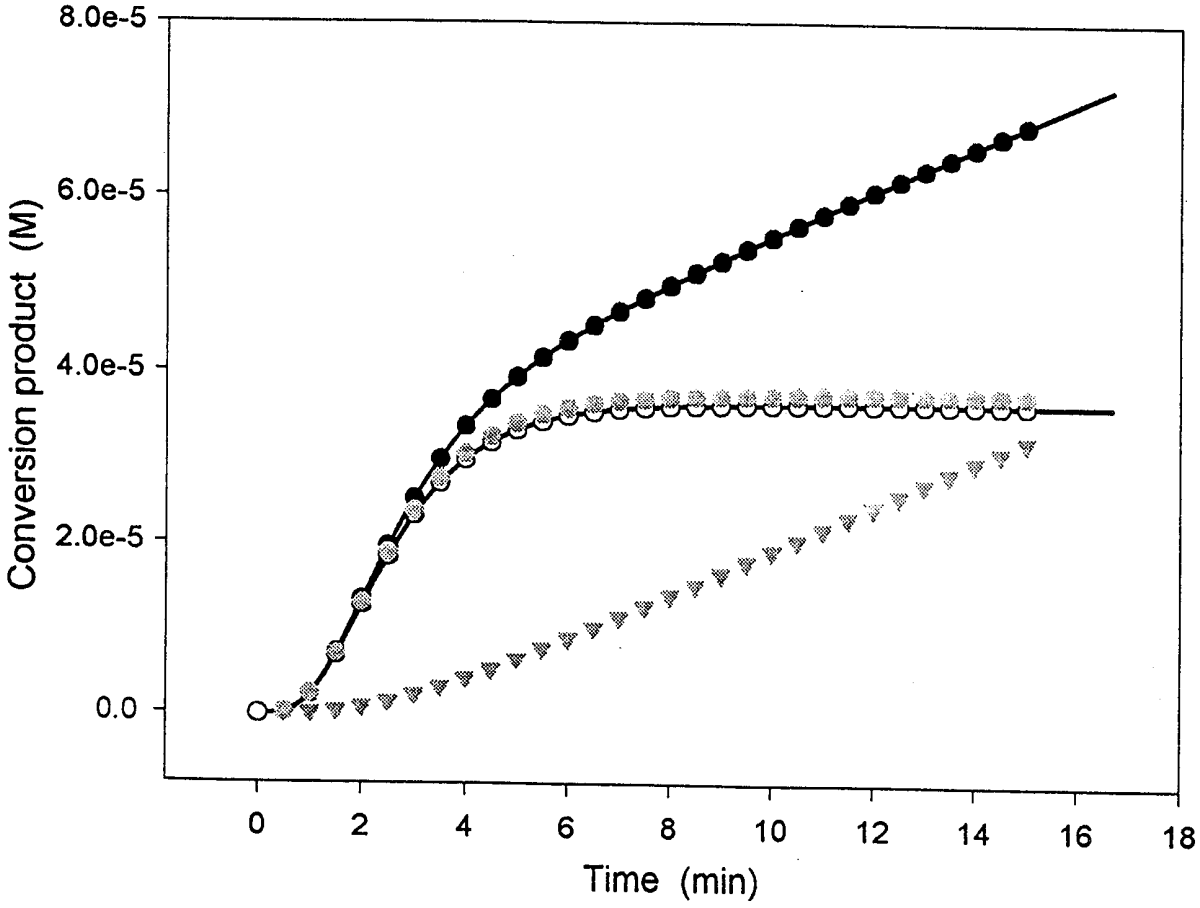

8.0e-5
6.0e-5
4.0e-5
2.0e-5
0.0
Conversion product (M)
0
2
4
6
8
10
12
14
16
18
Time (min)

Figure 1

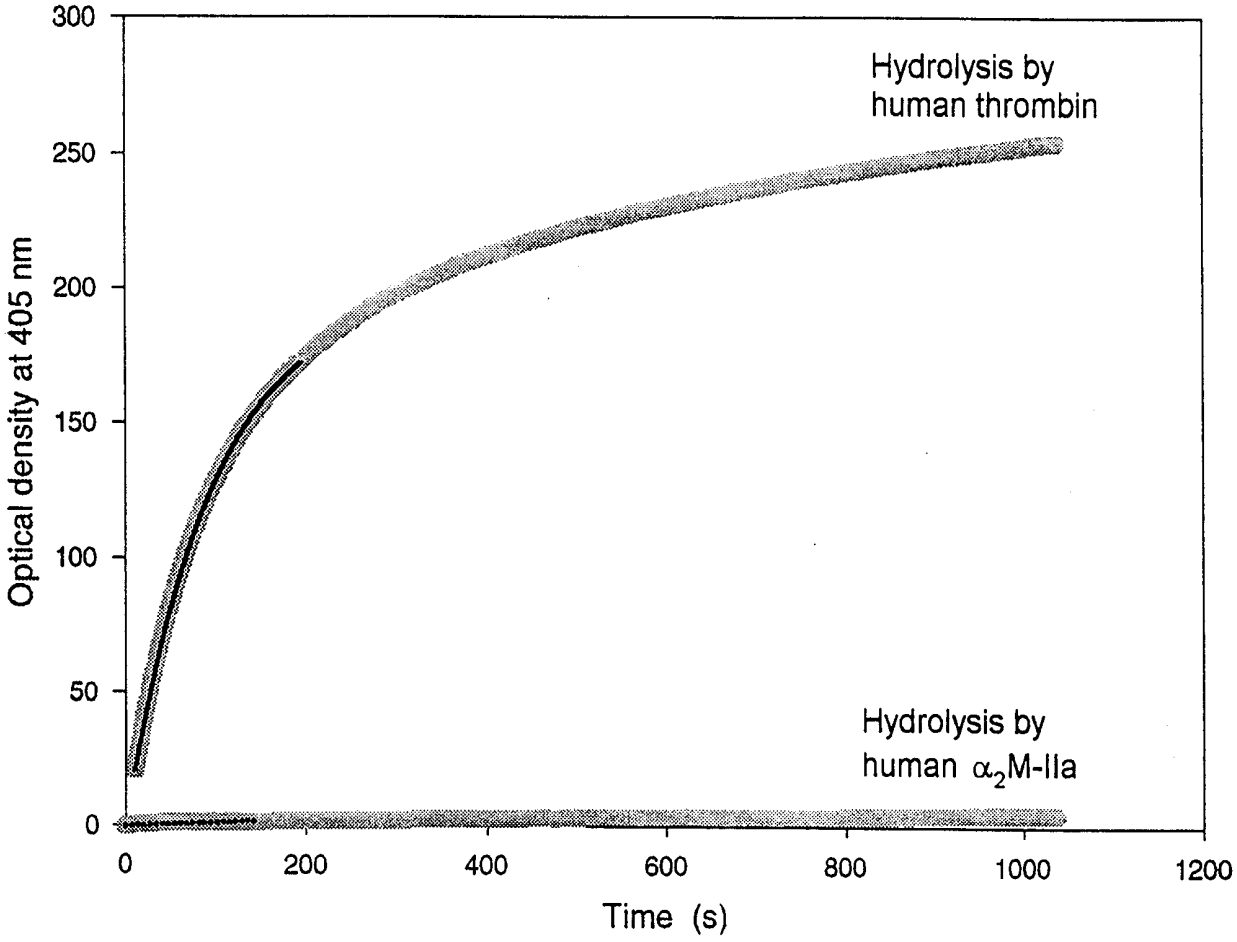

300
250
200
150
100
50
0
Optical density at 405 nm
0
200
400
600
800
1000
1200
Time (s)
Hydrolysis by human thrombin
Hydrolysis by human α_2M-IIa

Figure 2

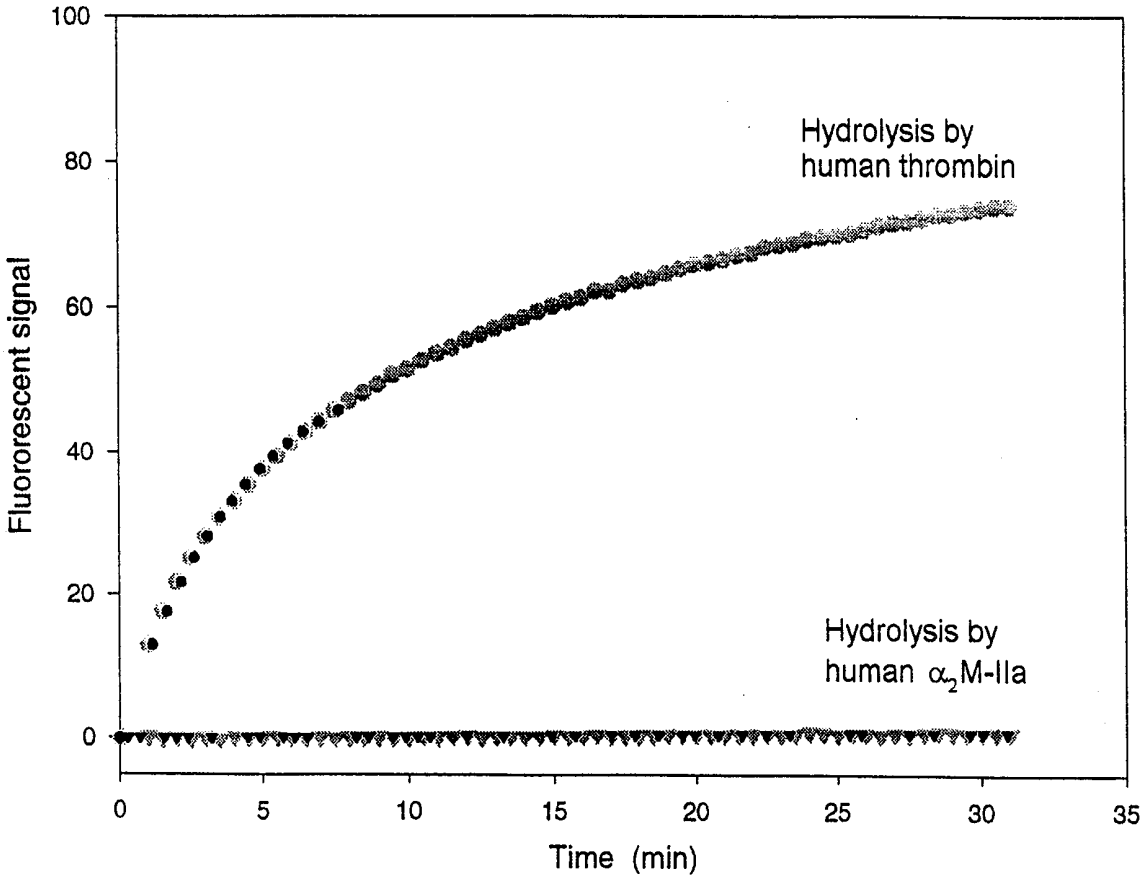

100
80
60
40
20
0
Fluorescent signal
0
5
10
15
20
25
30
35
Time (min)
Hydrolysis by human thrombin
Hydrolysis by human α_2M-IIa

Figure 3

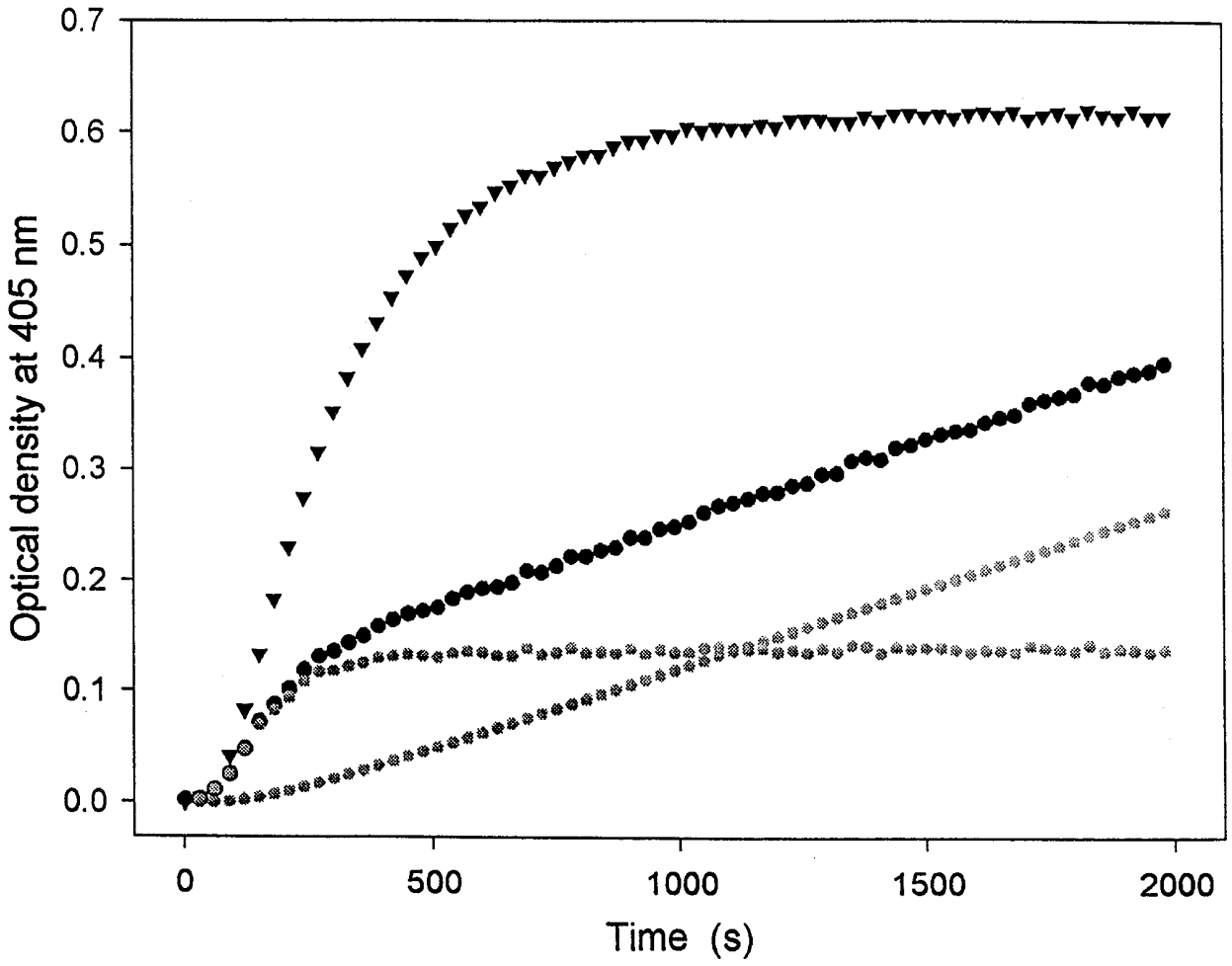

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Optical density at 405 nm
0
500
1000
1500
2000
Time (s)

Figure 4

INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/EP 00/01912

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/56 G01N33/86 C12Q1/37

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, MEDLINE, CHEM ABS Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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A	US 4 897 444 A (BRYNES PAUL J ET AL) 30 January 1990 (1990-01-30) the whole document ---	1-14
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 July 2000	14/07/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Hart-Davis, J

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INTERNATIONAL SEARCH REPORT

Inte .ional Application No
PCT/EP 00/01912

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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PCT/EP 00/01912

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