The invention relates to variants of plasminogen and plasmin comprising one or more point mutations in the catalytic domain which reduce or prevent autocatalytic destruction of the protease activity of plasmin. Compositions, uses and methods of using said variants of plasminogen and plasmin are also disclosed.
PLASMINOGEN AND PLASMIN VARIANTS

FIELD OF THE INVENTION

The invention relates to variants of plasminogen and plasmin comprising one or more point mutations in the catalytic domain which reduce or prevent autocatalytic destruction of the protease activity of plasmin. Compositions, uses and methods of using said variants of plasminogen and plasmin are also disclosed.

BACKGROUND TO THE INVENTION

Activation of the zymogen plasminogen results in the formation of the fibrinolytically/thrombolytically active serine proteinase plasmin. Activation of endogenous plasminogen can be triggered or enhanced by the administration of a plasminogen activator such as urokinase, streptokinase, staphylokinase or tPA, or any variant thereof. Upon activation, the plasminogen protein is proteolytically cleaved into a heavy chain comprising the 5 kringle domains and a light chain comprising the catalytic domain. Both chains are held together via 2 disulfide bonds. After activation, an autolytic cleavage removes an N-terminal segment from the heavy chain (78 amino acids of human plasmin; 77 amino acids of bovine plasmin) and the bovine plasmin heavy chain can be further autocatalytically cleaved between kringles 3 and 4, hence giving rise to bovine midiplasmin (Christensen et al. 1995, Biochem J 305, 97-102).

Activation of plasminogen to plasmin, triggered by the cleavage of the R561-V562 peptide bond in human plasminogen, induces a large conformational change in the light chain, said change resulting in the priming, or activation, of the catalytic triad within said light chain. Bacterial plasminogen activators such as streptokinase and staphylokinase form a complex with plasminogen and, without cleavage of the R561-V562 peptide bond of plasminogen, the catalytic site of plasminogen is activated due to conformational changes upon activator-plasminogen complex formation (plasminogen activation mechanisms are summarized in, e.g., the Introduction section of Terzyan et al. 2004; Proteins 56: 277-284).

Whereas plasminogen activators act as indirect thrombolytic agents, it has alternatively been suggested to use plasmin itself as a direct fibrinolytic/thrombolytic agent. Such direct use is, however, hampered by the fact that plasmin is, like many proteases, subject to autocatalytic
proteolytic degradation which follows second order kinetics subject to product inhibition (Jespersen et al. 1986, Thrombosis Research 41, 395-404).

In the early 1960's it was established that plasmin can be stabilized at acidic pH, or alternatively at neutral pH provided an amino acid such as lysine is present. Nevertheless, autolytic cleavage after Lys104, Arg189 and Lys622 (numbering relative to Lys-plasmin) were reported even when plasmin is stored at pH 3.8 (WO01/36608). When plasmin is stored at the even lower pH of 2.2, non-autolytic acid cleavage occurs between Asp-Pro (D-P) at postions Asp62, Asp154 and Asp346 (WO01/36608). This illustrates that pH can be lowered to a point where no apparent autocatalytic degradation occurs anymore but at which acid hydrolysis is becoming a factor of destabilization. No information is present in WOO1/36608 as to which peptide bonds in plasmin are vulnerable to (autocatalytic) hydrolysis at neutral pH. Known stabilizers of plasmin include glycerol, sufficiently high ionic strength, fibrinogen and ε-aminocaproic acid (EACA), as disclosed by Jespersen et al. (1986, Thromb Res 41, 395-404). Lysine and lysine-derivatives (such as EACA and tranexamic acid) and p-aminomethylbenzoic acid (PAMBA) are some further known stabilizers (Uehsima et al. 1996, Clin Chim Acta 245, 7-18; Verstraete 1985, Drugs 29, 236-261). US 4,462,980 reported on the formation of plasmin aggregates contributing to plasmin degradation despite storage at acidic conditions. A solution to this problem was provided in US 4,462,980 by means of adding a polyhydroxy compound. Other ways of stabilizing plasmin include the addition of oligopeptidic compounds (e.g. US 5,879,923). Alternatively, the catalytic site of plasmin can be reversibly blocked by means of derivatization, e.g. acylation (EP 0009879). Pegylation of plasmin has also been suggested as a means to stabilize the enzyme (WO 93/15189).

A number of plasmin variants other than truncated forms of plasmin have been described and include a chimeric microplasmin (WO 2004/045558) and variants with a point mutation at the two-chain cleavage site (US 5,087,572) or at a catalytic triad amino acid (Mhashilkar et al. 1993, Proc Natl Acad Sci USA 90, 5374-5377; Wang et al., 2001, J Mol Biol 295, 903-914). Wang et al. (1995, Protein Science 4, 1758-1767 and 1768-1779) reported an extensive series of microplasminogen mutants at amino acid positions 545, 548, 550, 555, 556, 558, 560-564, 585, 740 and 788. A double mutant wherein cysteines at amino acid positions 558 and 566 were
substituted for serines was reported by Linde et al. (1998, Eur J Biochem 251, 472-479). Takeda-Shitaka et al. (1999, Chem Pharm Bull 47, 322-328) refer to a plasmin variant with reduced activity, the variation involving the substitution of alanine at amino acid position 601 to threonine. All amino acid positions referred to above are relative to Glu-plasminogen starting with Glu at amino acid position 1. A non-cleavable plasminogen variant (cleavage between heavy and light chain impaired) is described in WO 91/08297. Dawson et al. (1994, Biochemistry 33, 12042-12047) describe the reduced affinity for streptokinase of a Glu-plasminogen variant with a Glu instead of Arg at position 719 (R719E). Jespers et al. (1998, Biochemistry 37, 6380-6386) produced in an Ala-scan the series of phage-displayed microplasminogen single-site mutants H569A, R610A, K615A, D660A, Y672A, R712A, R719A, T782A, R789A, and found that arginine at position 719 is key for interaction with staphylokinase; the D660A mutant was not further characterized due to very low expression; only the R719A mutant was additionally produced in soluble form. None of the mutants showed a gross change in proteolytic activity (substrate S-2403). Jespers et al. (1998) also included an active site mutant S741A in their analysis; the crystal structure of this mutant is disclosed in Wang et al. (2000, J Mol Biol 295, 903-914). In further attempts to unravel the streptokinase/plasminogen interaction sites, Terzyan et al. (2004, Proteins 56, 277-284) reported a number of microplasminogen mutants (K698M, D740N, S741A) in an already mutated background (R561A), the latter prohibiting proteolytic activation of plasminogen and thus prohibiting formation of active microplasmin (which would complicate the study of the contact-activation mechanism of the streptokinase-microplasminogen complex). Terzyan et al. (2004) further mention an "inadvertent" triple mutant R561A/H569Y/K698M apparently functionally indifferent from the double mutant R561A/K698M. Wang et al. (2000, Eur J Biochem 267, 3994-4001), in studying streptokinase/plasmin(ogen) interaction, produced a set of microplasminogen (amino acids 530-791 of Glu-plasminogen) mutants in a Cys536Ala and Cys541Ser background. These mutants include the R561A mutation as described above (Terzyan et al. (2004)) as well as R561A/K698G, R561A/K698A and R561A/K698Q double mutants. In the same C536A/C541S background, single K698G and K698A mutations were introduced also, of which the K698G was not characterized further due to difficulties with purification. The above studies aimed at obtaining a better understanding of the characteristics of the plasminogen/plasmin molecule and did not report any clinical usefulness or benefit or putative
clinical advantages of the plasminogen/plasmin mutants. Peisach et al. (1999, Biochemistry 38, 11180-11188) succeeded in determining the crystal structure of microplasminogen containing the M585Q, V673M and M788L mutations.

Nguyen & Chrambach (1981, Preparative Biochem 11, 159-172) reported the presence of "a minor and unidentified protein component" of 10.0 kDa based on reducing SDS-PAGE of a crude commercial preparation of urokinase-activated plasmin (Homolysin). The differences in autolysis of human plasmin depending on pH have been described in detail by Shi & Wu (1988, Thrombosis Research 51, 355-364). Ohyama et al. (2004, Eur J Biochem 271, 809-820) proposed the use of non-lysine analog plasminogen modulators in treatment of cancer due to the enhancement of plasmin autoproteolysis by such compounds which results in the enhanced formation of angiostatins (in the presence of the plasminogen activator urokinase). Table 3 of Ohyama et al. (2004) lists as many as 15 cleavage sites within plasmin subjected to autoproteolysis-enhancing compounds. In discussing their observations in view of prior investigations, it would seem that the autoproteolysis-enhancing compounds are more or less selectively enhancing proteolysis of the B/light-chain whereas minimum degradation of both A/heavy- and B/light-chain was found in the absence of the autoproteolysis-enhancing compounds.

It is clear that none of the above methods/variants solves the problem of providing a plasmin stabilized at the molecular level. The provision of a plasmin variant (or of a corresponding plasminogen variant from which plasmin can be derived) with a catalytic domain intrinsically resistant to autocatalytic degradation would be a significant step forward towards efficient and safe long-term storage as well as towards efficient and safe therapeutic use of plasmin such as in thrombolytic therapy or in the induction of posterior vitreous detachment or vitreous liquefaction in the eye.

SUMMARY OF THE INVENTION

The invention relates to isolated plasminogen variants or plasmin variants obtainable therefrom, or to isolated plasmin variants, or proteolytically active or reversibly inactive derivatives of any of said plasmin variants, wherein said variants are comprising an activation site and a catalytic
domain, characterized in that said catalytic domain contains a mutation of one or more amino acids at positions 1 to 4 of the human plasmin catalytic domain or at positions corresponding thereto in a non-human plasmin catalytic domain, wherein said human plasmin catalytic domain is starting with the amino acid valine at position 1 which is the same valine amino acid occurring at position 562 of human Glu-plasminogen. More in particular, if said catalytic domain is mutated at position 1, (i) the amino acid at position -1 relative to the plasmin catalytic domain is an arginine, lysine or other amino acid that maintains functionality of the activation site, (ii) the amino acid at position 24 of the human plasmin catalytic domain, or at the corresponding position of a non-human plasmin catalytic domain, is a methionine, and (iii) the amino acid at position 1 is mutated into an amino acid different from glycine or proline. Alternatively, if said catalytic domain is mutated at positions 1 and 2, the amino acid at position 24 of the human plasmin catalytic domain, or at the corresponding position of a non-human plasmin catalytic domain, is a methionine.

The mutation or mutations in the plasminogen variants, plasmin variants, or plasmin derivatives according to the invention reduce the extent of autoproteolytic degradation of said plasmin variant compared to the extent of autoproteolytic degradation of wild-type plasmin, such as determined with a chromogenic or biological substrate activity assay.

The plasminogen variants, plasmin variants, or plasmin derivatives according to the invention may be Glu-plasminogen or Glu-plasmin, Lys-plasminogen or Lys-plasmin, midiplasminogen or midiplasmin, miniplasminogen or miniplasmin, microplasminogen or microplasmin, deltaplasminogen or deltaplasmin.

The plasminogen variants, plasmin variants, or plasmin derivatives according to the invention are of particular interest for use as a medicament and can optionally by comprised and/or combined in a composition further comprising at least one of a pharmaceutically acceptable diluent, carrier or adjuvant. Such compositions may further comprise one or more of an anticoagulant, a thrombolytic agent, an anti-inflammatory agent, an antiviral agent, an antibacterial agent, an antifungal agent, an anti-angiogenic agent, an anti-mitotic agent, an antihistamine or an anaesthetic.
The invention further relates to methods for screening for autoproteolytically stable plasmin variants, said methods comprising:

(i) providing a plasmin variant according to the invention, and providing wild-type plasmin,
(ii) comparing the autoproteolytic stability of the variant plasmin and wild-type plasmin provided in (i), and
(iii) selecting from (ii) a variant which retains proteolytic activity, and of which autoproteolytic stability is increased relative to the autoproteolytic stability of wild-type plasmin.

The invention further relates to methods for producing a plasminogen variant according to the invention, said method including the steps of:

(i) introducing a nucleic acid encoding a plasminogen according to the invention in a suitable host cell capable of expressing said plasminogen;
(ii) growing the host cell obtained in (i) under conditions and during a time sufficient for expression of said plasminogen in said host cell; and
(iii) harvesting the plasminogen expressed in (ii).

The invention further relates to methods for producing a plasmin variant according to the invention, said method including the steps of:

(i) introducing a nucleic acid encoding a plasminogen according to the invention in a suitable host cell capable of expressing said plasminogen;
(ii) growing the host cell obtained in (i) under conditions and during a time sufficient for expression of said plasminogen in said host cell;
(iii) harvesting the plasminogen expressed in (ii);
(iv) activating the plasminogen of (iii) to plasmin.

The invention also relates to isolated nucleic acid sequences encoding a plasminogen variant or plasmin variant according to the invention, as well as to recombinant vector comprising such nucleic acid. Host cells transformed with the above nucleic acid or vector likewise are part of the invention.

FIGURE LEGENDS
FIGURE 1. Amino acid sequence with double numbering of the amino acid positions of wild-type human Glu-plasminogen (1 to 791) and of the plasmin catalytic domain (1 to 230, amino acid sequence and numbering in bold). Microplasminogen as used for demonstrating the invention starts at amino acid position 543 (numbering relative to Glu-plasminogen). Kringle domains (as derived from the information included in GenBank accession number AAA36451) are boxed and their amino acid sequences typed alternating in normal and italic letters. The catalytic triad amino acids are circled.

FIGURE 2. Amino acid sequence alignment of mammalian plasminogen proteins retrieved from GenBank. The sequence alignment was run with the COBALT software (Constraint-based Multiple Alignment Tool; Papadopoulos & Agarwala, Bioinformatics 23:1073-79, 2007) available through the National Center for Biotechnology Information (NCBI) website with default settings. ▼: indication of start of Glu-plasminogen. The amino acid numbering is relative to human plasminogen.

FIGURE 3. Picture (x 10) of posterior vitreous detachment induced after 5 days post-injection by 30 ng of the microplasmin Vail Ile variant.

DETAILED DESCRIPTION OF THE INVENTION

The current invention is based on the results of studying the effect of point mutations in the plasmin molecule, and more specifically in the catalytic domain of the plasmin molecule, on autoproteolysis. Peptide bonds susceptible to cleavage by plasmin are located at the C-terminus of lysine or arginine (Weinstein & Doolittle, 1972, Biochim Biophys Acta 258, 577-590). Nearly 10% (22 out of 230) of the amino acids of the plasmin catalytic domain (starting at amino acid position 562, a valine, in human Glu-plasminogen) are lysines or arginines. Theoretically all peptide bonds C-terminal of these lysines and arginines, independent of the structure of the amino acid C-terminal of said lysine or arginine, in one plasmin molecule can be proteolytically cleaved by another plasmin molecule. Further theoretically, the mutation of any one or more of these lysines or arginines into a non-lysine non-arginine amino acid would render a plasmin molecule more resistant to autoproteolytic degradation. This theory was proven to be correct, as
described in International Patent Publication No. WO2011/004011. Basis for the current invention is the unexpected observation that mutation of a wild-type amino acid located at the N-terminus of the catalytic domain, i.e. of an amino acid at positions 1 to 4 of the catalytic domain, into a non wild-type amino acid, greatly increases the resistance of the resulting mutant plasmin to autoproteolytic degradation, in conjunction with retaining proteolytic capacity by the mutant plasmin.

The invention relates to isolated plasminogen variants or plasmin variants obtainable therefrom, or to isolated plasmin variants, or proteolytically active or reversibly inactive derivatives of any of said plasmin variants, wherein said variants are comprising an activation site and a catalytic domain, characterized in that said catalytic domain contains a mutation of one or more amino acids at positions 1 to 4 of the human plasmin catalytic domain or at positions corresponding thereto in a non-human plasmin catalytic domain, wherein said human plasmin catalytic domain is starting with the amino acid valine at position 1 which is the same valine amino acid occurring at position 562 of human Glu-plasminogen. More in particular, if said catalytic domain is mutated at position 1, (i) the amino acid at position -1 relative to the plasmin catalytic domain is an arginine, lysine or other amino acid that maintains functionality of the activation site, (ii) the amino acid at position 24 of the human plasmin catalytic domain, or at the corresponding position of a non-human plasmin catalytic domain, is a methionine, and (iii) the amino acid at position 1 is mutated into an amino acid different from glycine or proline. Alternatively, if said catalytic domain is mutated at positions 1 and 2, the amino acid at position 24 of the human plasmin catalytic domain, or at the corresponding position of a non-human plasmin catalytic domain, is a methionine. In particular, the above plasminogen variant, plasmin variant, or plasmin derivative comprises the mutation of the amino acid valine at position 1 of the catalytic domain into isoleucine.

The mutation or mutations in the plasminogen variants, plasmin variants, or plasmin derivatives according to the invention reduce the extent of autoproteolytic degradation of said plasmin variant compared to the extent of autoproteolytic degradation of wild-type plasmin, such as determined with a chromogenic or biological substrate activity assay.
Mutations other than the one or those described above may also be present in the plasmin catalytic domain, as is illustrated in the Examples section.

The plasminogen variants, plasmin variants, or plasmin derivatives according to the invention may be Glu-plasminogen or Glu-plasmin, Lys-plasminogen or Lys-plasmin, midiplasminogen or midiplasmin, miniplasminogen or miniplasmin, microplasminogen or microplasmin, deltaplasminogen or deltaplasmin.

A mutation of an amino acid at a given position into a "non wild-type amino acid", or into an "amino acid different from the natural amino acid", is considered to be a change of the amino acid at said given position of a wild-type plasminogen or plasmin into any amino acid different from the wild-type or natural amino acid at that said given position of that said wild-type plasminogen or plasmin. Some considerations concerning the choice of the mutations follow further.

A person skilled in the art will be able to decide easily into which other amino acid a wild-type amino acid can be mutated. Such decision may, but must not necessarily imply, criteria such as amino acid size, amino acid charge, amino acid polarity, and/or amino acid hydrophobicity index (see Table 1). Moreover, the availability of the crystal structure of plasminogen and microplasmin (MMDB ID: 12717; PDB ID: 1DDJ; Wang et al., 2001, J Mol Biol 295, 903-914) is of great value in helping identifying the mutant amino acids such that the resulting mutant plasmin or plasminogen molecule retains proteolytic activity. Furthermore, it can be expected that mutation of a wild-type amino acid at a given position \([P+/-n]\), and optionally additionally at one or more of a given position \(P, P', P''\), etc., into either one of the amino acids of a given group will yield similar results. Based on Table 1, said given groups can be defined as follows:

- hydrophobic aliphatic amino acids: Met, Ile, Leu and Val
- hydrophobic aromatic amino acids: Phe
- hydrophilic acidic amino acids: Asp, Glu, Asn and Gin
- hydrophilic basic amino acids: Arg, Lys and His
- moderately hydrophobic aliphatic amino acids: Gly, Ala, Ser, Thr, Cys, Pro
- moderately hydrophobic aromatic amino acids: Tyr and Trp.
Of these, and for the purpose of mutation, Cys and Pro may be less favorable substitute amino acids of wild-type plasmin or plasminogen amino acids due to the creation of possible free thiol-group by a Cys, or due to more extensive disturbance of the protein structure by a Pro. Other amino acid substitutions include the mutation of a wild-type amino acid at a position \([P+/-n]\), and optionally additionally at one or more of a position P, P', P", etc., of a plasmin(ogen) catalytic domain into a non-natural or noncanonical amino acid, or into amino acid analogs, such as norleucine, norvaline, ornithine or citrulline (for more extensive list see, e.g., Hendrickson et al. 2004, Annu Rev Biochem 73, 147-176).

### Table 1. Characteristics of amino acids.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Side chain polarity</th>
<th>Side chain charge (at pH 7)</th>
<th>Hydropathy index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>Ala</td>
<td>nonpolar</td>
<td>neutral</td>
</tr>
<tr>
<td>Arginine</td>
<td>Arg</td>
<td>polar</td>
<td>positive</td>
</tr>
<tr>
<td>Asparagine</td>
<td>Asn</td>
<td>polar</td>
<td>neutral</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>Asp</td>
<td>polar</td>
<td>negative</td>
</tr>
<tr>
<td>Cysteine</td>
<td>Cys</td>
<td>nonpolar</td>
<td>neutral</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>Glu</td>
<td>polar</td>
<td>negative</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Gln</td>
<td>polar</td>
<td>neutral</td>
</tr>
<tr>
<td>Glycine</td>
<td>Gly</td>
<td>nonpolar</td>
<td>neutral</td>
</tr>
<tr>
<td>Histidine</td>
<td>His</td>
<td>polar</td>
<td>positive</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>Ile</td>
<td>nonpolar</td>
<td>neutral</td>
</tr>
<tr>
<td>Leucine</td>
<td>Leu</td>
<td>nonpolar</td>
<td>neutral</td>
</tr>
<tr>
<td>Lysine</td>
<td>Lys</td>
<td>polar</td>
<td>positive</td>
</tr>
<tr>
<td>Methionine</td>
<td>Met</td>
<td>nonpolar</td>
<td>neutral</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>Phe</td>
<td>nonpolar</td>
<td>neutral</td>
</tr>
<tr>
<td>Proline</td>
<td>Pro</td>
<td>nonpolar</td>
<td>neutral</td>
</tr>
<tr>
<td>Serine</td>
<td>Ser</td>
<td>polar</td>
<td>neutral</td>
</tr>
<tr>
<td>Threonine</td>
<td>Thr</td>
<td>polar</td>
<td>neutral</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Trp</td>
<td>nonpolar</td>
<td>neutral</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Tyr</td>
<td>polar</td>
<td>neutral</td>
</tr>
<tr>
<td>Valine</td>
<td>Val</td>
<td>nonpolar</td>
<td>neutral</td>
</tr>
</tbody>
</table>

The identification of an amino acid in a non-human plasmin(ogen) sequence which "corresponds to" (i.e. the identification of a "corresponding" amino acid) an amino acid in the human plasmin(ogen) first implies the alignment of both amino acid sequences. Such alignment may require some optimization, such as introduction of minor gaps in one or both of the aligned sequences, to result in the highest identity and homology. Secondly, the amino acid in the non-
human plasmin(ogen) aligning with the amino acid in the human plasmin(ogen) is identified and is herein referred to as the "corresponding" amino acid. Figure 2 herein depicts such an alignment of publicly available mammalian plasminogen protein sequences, and highlights the amino acids of particular interest to the current invention in the human plasminogen sequence (line 1) together with the corresponding amino acids in the non-human plasminogen sequences (lines 2-18). The amino acids P, P', etc., of particular interest are Lys at position 698 (position 137 in the catalytic domain, see Figure 1), Lys at position 708 (position 147 in the catalytic domain, see Figure 1) and Arg at position 719 (position 158 in the catalytic domain, see Figure 1).

"Plasmin", also known as fibrinolysin or lysofibrin, is a serine-type protease which results from the activation of the zymogen plasminogen. Activation is the result of a proteolytic cleavage between amino acids 561 and 562 (numbering relative to human Glu-plasminogen). Plasmin carries a heavy chain comprising 5 kringle domains and a light chain comprising the catalytic domain. Plasminogen can be enriched from blood plasma, e.g., via lysine affinity-chromatography (Deutsch & Mertz, 1970, Science 170, 1095-1096). Truncation of the plasmin molecule (outside and/or inside the plasmin catalytic domain) is possible as long as the catalytic domain remains functional, such truncation thus results in the formation of a "proteolytically active derivative" of plasmin. As such, one or more of the 5 kringle domains can be deleted wholly or partially. Truncated plasmins lacking one or more kringle domains and/or lacking parts of one or more kringle domains therefore are envisaged by the current invention as examples of proteolytically active derivatives of plasmin. Examples of truncated variants of plasmin include, but are not limited to, "midiplasmin", "miniplasmin", "microplasmin", and "delta-plasmin". Midiplasmin is basically lacking kringle domains 1 to 3 (e.g. Christensen et al., 1995, Biochem J 305, 97-102). Miniplasmin was originally obtained by limited digestion of plasmin with elastase and is basically lacking kringle domains 1 to 4 (e.g. Christensen et al., 1979, Biochim Biophys Acta 567, 472-481; Powell & Castellino, 1980, J Biol Chem 255, 5329). Miniplasmin has subsequently been produced recombinantly (WO 2002/050290). Microplasmin was originally obtained by incubation of plasmin at elevated pH and is basically lacking all kringle domains (e.g. WO 89/01336). Whereas the microplasmin obtained from incubation of plasmin at elevated pH is containing the 30-31 carboxy-terminal amino acids of the heavy chain, a recombinantly
produced microplasmin variant is containing the 19 carboxy-terminal amino acids of the heavy chain (WO 2002/050290). This illustrates the allowed molecular variability within a given plasmin genus such as the microplasmin genus (e.g. multiple species form the microplasmin genus). Delta-plasmin is a recombinant version of plasmin in which kringle domain 1 is linked directly with the catalytic domain (WO 2005/105990). The above described truncated variants of plasmin are obtained by activation of "midiplasminogen", "miniplasminogen", "microplasminogen" and "delta-plasminogen", respectively. In order to be activatable, a truncated plasminogen needs to comprise a minimum number of amino acids of the linker between the kringle domain (such as kringle 5 domain in miniplasmin) and the catalytic domain, or C-terminal of the catalytic domain in case of a kringle-less truncated plasmin (see, e.g., Wang et al., 1995, Protein Science 4, 1758-1767). In the context of the present invention it may be desired that the plasminogen comprises an "intact activation site", which implies that at least amino acids 561 and 562 (relative to human Glu-plasminogen; or the corresponding amino acids in non-human plasminogen) are such that activation/conversion of plasminogen to plasmin can occur, albeit possibly with different kinetics, as it occurs in wild-type plasmin. As alternative to plasmin or an active truncated variant thereof, an activatable plasminogen or a truncated variant thereof can be used in the context of the current invention (see, e.g. EP 0480906; US 5,304,383; EP 0631786; US 5,520,912; US 5,597,800; US 5,776,452). "Plasminogen" refers to any form of plasminogen e.g. Glu-plasminogen or Lys-plasminogen (starting with Arg at position 68 or Lys at positions 77 or 78). When using activatable plasminogen or an activatable truncated variant thereof, the activation to plasmin may be delayed and will typically occur after contacting it with an organ, tissue or body fluid, i.e. after administration to a subject. In yet another alternative, the plasmin or an active truncated variant thereof can be substituted in the context of the current invention for an activatable plasminogen or an activatable truncated variant thereof in conjunction with a plasminogen activator (such as tissue plasminogen activator (tPA), urokinase, streptokinase or staphylokinase, or any variant thereof; see, e.g. US 6,733,750; US 6,585,972; US 6,899,877; WO 03/33019). In yet a further alternative, a mixture of any of (i) plasmin or derivative thereof, (ii) activatable plasminogen or an activatable derivative thereof, and, optionally (iii) a plasminogen activator can be used in the context of the current invention (see, e.g. US 2004/0081643). In order to ensure stability of the plasmin (or plasminogen), it will generally be stored at lowered temperatures (e.g. +4 degrees Celsius or -20 degrees Celsius). The
storage composition may be a stabilizing composition such as a low pH composition (pH 4 or lower; obtained by e.g. 1 mM to 250 mM of an acid such as citric acid, see, e.g. Castellino & Sodetz, 1976, Methods Enzymol 45, 273-286; WO 01/36608; WO 01/36609; WO 01/3661) or a high glycerol content composition (30-50% v/v, e.g., Castellino & Sodetz, 1976, Methods Enzymol 45, 273-286), alternatively in or in conjunction with one or more further stabilizer compositions comprising e.g. an amino acid (e.g. lysine or an analogue thereof such as EACA or tranexamic acid), a sugar (e.g. mannitol) or any stabilizer as known in the art (e.g. dipeptides, WO 97/01631). Further included in the genus "plasmin" is any active derivative thereof (or of an active truncated plasin variant), or similar derivative of activatable plasminogen (or of activatable truncated variant thereof). Such derives include e.g. labeled plasmin or plasminogen (or truncated variants thereof) such as Tc"-labeled plasmin (Deacon et al., 1980, Br J Radiol 53, 673-677) or pegylated or acylated plasmin or plasminogen (or truncated variants thereof; EP 9879, WO 93/15189). Any other label (radioactive, fluorescent, etc.) may also be used to produce a plasmin or plasminogen derivative. Said derivatives further include hybrid or chimeric plasmin or plasminogen molecules comprising e.g. a truncated plasmin or plasminogen according to the invention fused with e.g. a fibrin-binding molecule (such as kringle 2 of tPA, an apolipoprotein kringle, the finger domain of tPA or fibronectin or the Fab domain of a fibrin-binding antibody).

Comparison of the autoproteolytic resistance (i.e. stability) of wild-type plasmin and of plasmin variants or plasmin derivatives according to the invention can be performed in a similar way as for comparing proteolytic activity, e.g., in a chromogenic activity assay or a biological substrate assay based on e.g. fibrin, fibrinogen, fibronectin, gelatin, laminin or collagen.

In order to determine autoproteolytic resistance, the autolysis rate constant can be determined. It is envisaged that the plasmin variants according to the invention, including the plasmins obtained from the plasminogen variants according to the invention, or any of the plasmin derivatives according to the invention may be characterized by an autolysis rate constant that is at least 5%, or at least 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 60%, 70%, 75%, 80%, 90%, 95%, 99% or 99.5% lower than the autolysis rate constant of wild-type plasmin, or, alternatively, by an autolysis rate constant that is at most 95%, or at most 0.5%, 1%, 5%, 10%, 15%, 20%,
25%, 30%, 35%, 40%, 45%, 50%, 60%, 70%, 75%, 80%, or 90% of the autolysis rate constant of wild-type plasmin. In order to determine the indicated percentage, the calculation can be done based on the absolute autolysis rate constant numbers. For example, an autolysis rate constant of 123 M⁻¹V¹ was determined for wild-type microplasmin, whereas for the microplasmin variant VII an autolysis rate constant of 33 M⁻¹V¹ was determined (see Examples). The autolysis rate constant of the VII variant therefore is 26.8% of the autolysis rate constant of wild-type microplasmin.

Further, any of the plasmin variants according to the invention, including the plasmins obtained from the plasminogen variants according to the invention, or derivatives of any of said plasmins may retain proteolytic activity different (higher or lower) from the proteolytic activity of wild-type plasmin, such as determined with e.g. a chromogenic activity assay or a biological substrate assay based on e.g. fibrin, fibrinogen, fibronectin, gelatin, laminin or collagen.

The proteolytic activities of the plasmin variants according to the invention, including the plasmins obtained from the plasminogen variants according to the invention, or any of the plasmin derivatives according to the invention may be compared to the proteolytic activity of wild-type plasmin by means of the catalytic constant k_{cat} which is a measure of the number of substrate molecule each enzyme site converts to product per unit time. Thus, any of the plasmin variants according to the invention, including the plasmins obtained from the plasminogen variants according to the invention, or any of the plasmin derivatives according to the invention may be characterized by a k_{cat} value which is in the range of +100% to -90%, or +50% to -50% of the k_{cat} value of wild-type plasmin, i.e., characterized by a k_{cat} value in the range of 10% to 200%, or 50% to 150% of the k_{cat} value of wild-type plasmin. In order to determine the indicated percentage, the calculation is done on the absolute k_{cat} numbers. For example, wild-type microplasmin has a k_{cat} of 46 s⁻¹, whereas the microplasmin variant K137M has a k_{cat} of 36 s⁻¹ (see Example 4/Table 3 of WO 2011/004011). The k_{cat} of the K137M variant therefore is 78.3% of the k_{cat} of wild-type microplasmin.

Another way of comparing proteolytic activity of the plasmin variants according to the invention, including the plasmins obtained from the plasminogen variants according to the invention, or any
of the plasmin derivatives according to the invention to proteolytic activity of wild-type plasmin includes comparing \( k_{cat}/K_m \). Although higher, comparable or slightly lower \( k_{cat}/K_m \) values may be preferred, an up to 1000-times or up to 500-times lower \( k_{cat}/K_m \) of a plasmin variants according to the invention, including the plasmins obtained from the plasminogen variants according to the invention, or any of the plasmin derivatives according to the invention compared to the \( k_{cat}/K_m \) of wild-type plasmin can still be acceptable (see further). By way of example, the \( k_{cat}/K_m \) of the VII microplasmin variant was determined to be \( 1 \times 10^6 \) whereas the \( k_{cat}/K_m \) of wild-type plasmin was determined to be \( 6.9 \times 10^5 \) (see Examples), i.e. the \( k_{cat}/K_m \) value of VII microplasmin is 1.45 times higher than the \( k_{cat}/K_m \) value of wild-type microplasmin.

Alternatively, any of the plasmin variants according to the invention, including the plasmins obtained from the plasminogen variants according to the invention, or any of the plasmin derivatives according to the invention may be compared to wild-type plasmin by combining autolytic rate constant data and \( k_{cat}/K_m \) data. For example, a plasmin variant with a 20-times lower autolytic rate constant compared to wild-type plasmin, and with a 10-times lower \( k_{cat}/K_m \) compared to wild-type plasmin will be 2-times better than the wild-type plasmin. Obviously depending on the ultimate use, a very stable plasmin (i.e. no or nearly no autoproteolytic degradation) with low proteolytic activity may be highly desired, e.g., in cases where low but prolonged plasmin activity is desired or even required to achieve the intended clinical effect. Such highly stable plasmin variants with low proteolytic activity would as such virtually equal slow-release formulations without the real need to actually use a slow-release carrier or adjuvant.

Yet another alternative to compare any of the plasmin variants according to the invention, including the plasmins obtained from the plasminogen variants according to the invention, or any of the plasmin derivatives according to the invention may be compared to wild-type plasmin by combining autolytic rate constant data and \( k_{cat} \) data.

Further, any of the plasmin variants according to the invention, including the plasmins obtained from the plasminogen variants according to the invention, or any of the plasmin derivatives according to the invention may be characterized by any combination of the above-defined autolysis rate constant, catalytic constant \( k_{cat} \) and/or \( k_{cat}/K_m \).
Obviously, for any comparative measurements such as described above it is desirable to compare plasmin variants with their closest wild-type plasmin, e.g., to compare a microplasmin variant with wild-type microplasmin, or a miniplasmin variant with wild-type miniplasmin. Furthermore obvious, for any activity measurement, a reversibly inactivated derivative of a plasmin variant according to the invention should first be activated by removing the cause of reversible inactivation (e.g. acylation or non-optimal pH).

Any of the plasminogen variants according to the invention or plasmins obtained therefrom, of the plasmin variants according to the invention may be Glu-plasminogen of Glu-plasmin, Lys-plasminogen or Lys-plasmin, midiplasminogen or midiplasmin, miniplasminogen or miniplasmin, microplasminogen or microplasmin, deltapiasminogen or deltapiasmin.

Many assays exist to determine whether or not a plasmin species is proteolytically active. Easy and straightforward assays are based on the digestion of a chromogenic substrate by plasmin present in a sample; chromogenic substrates include S-2403 (Glu-Phe-Lys-pNA) and S-2251 (Val-Leu-Lys-pNA) which release p-nitroaniline (pNA) upon proteolytic cleavage. The amount of pNA formed can be measured by light absorbance at 405nm. An alternative assay for determining plasmin activity is a potentiometric assay. Colorimetric (using a chromogenic substrate) and potentiometric assays are described in e.g., Castellino & Sodetz (1976, Methods Enzymol 45, 273-286). A further alternative assay for determining plasmin activity is a caseinolytic assay (e.g., Robbins & Summaria, 1970, Methods Enzymol 19, 184-199; Ruyssen & Lauwers, 1978, Chapter IX - Plasmin, In "Pharmaceutical Enzymes", Story-Scientia, Gent, Belgium, pp. 123-131). Yet another alternative assay for determining plasmin activity is a fibrinolytic assay (e.g., Astrup & Mullertz, 1952, Arch Biochem Biophys 40, 346-351). Further activity assays could be easily designed using other protein substrates. Clearly, such assays may also be used to follow disappearance of plasmin proteolytic activity over time due to autoproteolytic degradation of the enzyme. As an alternative for assessing stability of a plasmin variant or any active truncated variant or derivative thereof of the current invention, said plasmin variant may be incubated in the presence of wild-type plasmin and the resistance of the plasmin variant to digestion by wild-type plasmin can be monitored.
The use of plasmin in the removal of necrotic elements or debris from lesions, wounds, ulcerating wounds (such as ulcerating stitched wounds) etc. has been described in e.g. US 3,208,908. Similarly, topical application of plasmin-comprising therapeutic preparations for the treatment of burns was disclosed in e.g. US 4,122,158. Debridement refers to the removal of dead, damaged and/or infected tissue in order to improve or increase the healing of remaining healthy tissue. Such removal may be obtained by surgical, mechanical or chemical means, or by means of certain species of live maggots that selectively eat necrotic tissue (maggot therapy). Debridement may also be performed using enzymes or may be assisted by enzymes, a process referred to as enzymatic debridement. Debridement is an important aspect in the healing process of burns and other serious wounds and it is used as well in the treatment of some types of snake bites. The application of plasmin (or of any variant or derivative thereof or alternative therefore as described above) in enzymatic debridement (alone or in combination with other types of debridement) is particularly useful in promoting or facilitating wound healing and as an adjunct in surgical procedures such as skin grafting.

A more commonly known use of plasmin (or of any variant or derivative thereof or alternative therefore as described above) relates in general terms to the treatment of (a) pathological deposit(s) of fibrin. Fibrin deposits can result from a wide variety of pathological situations in the body. For example, fibrin-containing blood clots can form in vessels in tissue resulting in deep vein, coronary artery, cerebral artery or retinal vein occlusion or thrombosis. Small accumulations of fibrin precede, and may provide, warning of impending catastrophic thrombosis. Examples include unstable angina pectoris, which is considered a warning of impending coronary thrombosis and transient ischemic attacks, which may precede strokes. Fibrin is furthermore frequently deposited in tissue in association with inflammation associated with many disease processes including infection, autoimmune disease and cancer. Another situation where fibrin is deposited is around abscesses caused by infection with microorganisms. Fibrin deposits are furthermore frequently found associated with certain solid tumors. Fibrin deposition may also occur during the healing of any type of wound, including those resulting from surgical intervention, including e.g. trabeculectomy. Yet another situation of fibrin deposition is the accumulation of fibrin in a retinal vein, which can lead to retinal degeneration, disturbed vision or even loss of vision. The term pathological fibrin deposit further encompasses
such deposits as formed or as present in or at the tip of a catheter, catheter device or other implant such as prosthetic vessels and grafts of synthetic, human or animal origin and effectively blocked by an occlusion comprising fibrin. The term "catheter device" refers to any catheter or tube-like device that may enter the body, including arterial catheters, cardiac catheters, central venous catheters, intravenous catheters, peripherally inserted central catheters, pulmonary artery catheters, tunneled central venous catheters and arterio-venous shunts.

Among the various factors encouraging the process of thrombosis, i.e. the formation of a thrombus or hemostatic plug, are: (1) damage to the endothelial cell lining of the affected blood vessel, (2) an increase in the clotting properties of the blood, and (3) stagnation of blood in the affected blood vessel. Thrombosis can start as a very small lump attached to the damaged part of the blood vessel lining. Its presence encourages further thrombosis to occur, and has the effect of causing a slow-down of blood flow by reducing the inner diameter of the vessel. Further growth of the initially small thrombus often leads to total or almost total blockage of the affected blood vessel. If thrombosis takes place in one of the arteries, the tissues supplied by that artery may be deprived of oxygen and nutrition, causing damage or death of the tissue (gangrene). The severity of the damage depends upon the position and size of the thrombosis, the speed at which it grows and whether the affected area has only one artery or is supplied by collateral blood vessels. If the vessel to a vital organ is affected, e.g. the heart or the brain, the person may be severely crippled or die. Sometimes a thrombus may contain infective organisms such as bacteria, and septic thrombosis may occur, with the formation of pus and infection of the surrounding tissues.

Further uses of plasmin (or of any variant or derivative thereof or alternative therefore as described above) include the reduction of the level of circulating fibrinogen (e.g. WO 93/07893) and its use as an a2-antiplasmin inhibitor (reported to reduce the size of cerebral infarct after ischemic stroke; WO 00/18436).

Yet another use of plasmin (or of any variant or derivative thereof or alternative therefore as described above) is related to the induction of posterior vitreous detachment (PVD) and/or vitreous liquefaction in the eye as an alternative for or as adjunct to mechanical vitrectomy (WO 2004/052228; US 6,733,750; US 6,585,972; US 6,899,877; WO 03/33019; WO 2006/122249; WO 2007/047874; US 5,304,118; US 2006/0024349; US 2003/0147877). Vitrectomy and/or
vitreous liquefaction is of benefit for a number of eye conditions such as vitreous floaters (motile debris/deposits of vitreous within the normally transparent vitreous humour which can impair vision), retinal detachment (a blinding condition which may be caused by vitreal traction), macular pucker (scar tissue on macula; macula is required for sharp, central vision; macular pucker is also known as epi- or preretinal membrane, cellophane maculopathy, retina wrinkle, surface wrinkling retinopathy, premacular fibrosis, or internal limiting membrane disease), diabetic retinopathy (proliferative or non-proliferative) which may result in vitreal hemorrhage and/or formation of fibrous scar tissue on the retina (which may cause retinal detachment), macular holes (hole in macula causing a blind spot and caused by vitreal traction, injury or a traumatic event), vitreous hemorrhage (caused by diabetic retinopathy, injuries, retinal detachment or retinal tears, subarachnoidal bleedings (Terson syndrome), or blocked vessels), subhyaloid hemorrhage (bleeding under the hyaloid membrane enveloping the vitreous), macular edema (deposition of fluid and protein on or under the macula of the eye) and macular degeneration (starting with the formation of drusen; occurs in dry and wet form; if correlated with age coined age-related macular degeneration). Other eye-applications of plasmin include the maintenance or rescue of a filtering bleb after trabeculectomy surgery (performed to reduce intra-ocular pressure), see e.g. WO 2009/073457.

Another further use of plasmin (or of any variant or derivative thereof or alternative therefore as described above) resides in diagnosis, more particularly appropriately labeled (e.g. Tc"-labeled, see above) plasmin (or any variant or derivative thereof or alternative therefore as described above) may be applied for detecting pathological fibrin deposits. When applying a truncated plasmin or plasminogen variant according to the current invention in such diagnosis, care should be taken that said variant still comprises a fibrin-binding site (whether or not from plasmin itself or added to e.g. the plasmin catalytic domain by creating a hybrid molecule).

The plasmin or any variant or derivative thereof or alternative therefore according to the invention may be stored in a pharmaceutically acceptable carrier, diluent or adjuvant. Such carrier, diluent or adjuvant may consist of or comprise an acidic low buffer such as 1-100 mM acetate or citrate. When acidic, the pharmaceutically acceptable carrier, diluent or adjuvant may have a pH of 2.5 to 5.0, such as at pH of 2.5 to 4.0, or such as at a pH of 3.0 to 3.5, or such as a pH of 3.1. Useful acidic compounds include acetic acid, citric acid, hydrochloric acid, lactic acid,
malic acid, tartaric acid or benzoic acid. Formic acid may be used but care should be taken that this compound is not inducing proteolytic cleavage at the C-terminus of Asp-residues. The pharmaceutically acceptable carrier, diluent or adjuvant, when either acidic, neutral or basic, may comprise one or more amino acids such as serine, threonine, methionine, glutamine, glycine, isoleucine, valine, alanine, aspartic acid, lysine, histidine or any derivatives or analogues thereof.

The pharmaceutically acceptable carrier, diluent or adjuvant may comprise a carbohydrate such as a monosaccharide, disaccharide, polysaccharide or polyhydric alcohol. Examples include sugars such as sucrose, glucose, fructose, lactose, trehalose, maltose and mannose, sugar alcohols such as sorbitol and mannitol and polysaccharides such as dextrins, dextrans, glycogen, starches and cellulosics. The pharmaceutically acceptable carrier, diluent or adjuvant may comprise compounds such as glycerol, niacinamide, glucosamine, thiamine, citrulline, inorganic salts (such as sodium chloride, potassium chloride, magnesium chloride, calcium chloride), benzyl alcohol or benzoic acid. The pharmaceutically acceptable carrier, diluents or adjuvant may comprise compounds such as ε-aminocaproic acid (EACA) and/or tranexamic acid (see also above & Background section). Some of these compounds may be used as stabilizer of a plasmin or any variant or derivative thereof or alternative therefore as described above.

In view of the above, another aspect of the invention relates to the isolated plasminogen, plasmin, or any variant or derivative thereof or alternative therefore according to the invention, or a combination of any thereof for use as a medicament.

A further aspect of the invention relates to compositions comprising the isolated plasminogen, plasmin, or any variant or derivative thereof or alternative therefore according to the invention, or a combination of any thereof, and at least one of a pharmaceutically acceptable diluent, carrier or adjuvant. In a further embodiment, said composition may additionally comprise at least one of an anticoagulant, a further thrombolytic agent, an anti-inflammatory agent, an antiviral agent, an antibacterial agent, an antifungal agent, an anti-angiogenic agent, an anti-mitotic agent, an antihistamine or an anaesthetic.

In an embodiment to the above-described two aspects of the invention, the isolated plasminogen, plasmin, or any variant or derivative thereof or alternative therefore according to the invention,
or of a combination of any thereof, or the composition according to the invention may be used in any clinically relevant setting such as for treating a pathological fibrin deposit, for inducing posterior vitreous detachment in the eye, for inducing liquefaction of the vitreous in the eye, as adjunct to and facilitating vitrectomy in the eye, for inducing posterior vitreous detachment, for resolving vitreomacular adhesion, for closing macular holes, for enzymatic debridement, for reducing circulating fibrinogen, for reducing a2-antiplasmin levels, or in conjunction with trabeculectomy.

In another embodiment to the above-described two aspects of the invention, the isolated plasminogen, plasmin, or any variant or derivative thereof or alternative therefore according to the invention, or of a combination of any thereof, or the composition according to the invention may be used for prophylactic purposes or in methods for prophylactic treatment. Prophylactic uses include reducing the risk of development of a pathological fibrin deposit in a mammal having an increased risk of developing it (such as an obese mammal, a mammal not doing sufficient physical exercise or a mammal scheduled to undergo a major surgical event or operation). Other prophylactic uses include the induction of posterior vitreous detachment and/or vitreous liquefaction in an apparent healthy eye of a mammal of which the companion eye is/was diagnosed to require induction of posterior vitreous detachment and/or vitreous liquefaction.

Alternatively, the invention relates to methods for treating, dissolving, loosening, macerating, lysing, inducing or promoting lysis of a pathological fibrin deposit in a subject, said methods comprising contacting said fibrin deposit with an effective amount of the isolated plasminogen, plasmin, or any variant or derivative thereof or alternative therefore according to the invention, or of a combination of any thereof, said contacting resulting in the treatment, dissolution, loosening, maceration, lysis, or induction or promotion of lysis of said pathological fibrin deposit.

The invention further relates to methods for inducing posterior vitreous detachment in the eye and/or for inducing liquefaction of the vitreous in the eye, or for facilitating surgical vitrectomy in the eye in a subject, said methods comprising contacting an eye of said subject in need of such treatment with an effective amount of the isolated plasminogen, plasmin, or any variant or derivative thereof or alternative therefore according to the invention or of a combination of any
thereof, said contacting resulting in the induction of said posterior vitreous detachment and/or of said liquefaction of the vitreous, or in the facilitation of said surgical vitrectomy.

The invention also relates to methods for enzymatic debridement of injured tissue of a subject, said method comprising contacting said injured tissue with an effective amount of the isolated plasminogen, plasmin, or any variant or derivative thereof or alternative therefore according to the invention, or of a combination of any thereof, said contacting resulting in said enzymatic debridement of said injured tissue.

Other methods of the invention are treating or preventing any other clinically relevant indication, including methods for reducing circulating fibrinogen, or for reducing a2-antiplasmin levels in a subject, said methods comprising contacting a subject in need of such treatment with an effective amount of the isolated plasminogen, plasmin, or any variant or derivative thereof or alternative therefore according to the invention, or of a combination of any thereof, said contacting resulting in said reduction of circulating fibrinogen or of said a2-antiplasmin levels.

In general, the medicament or composition of the invention comprising a plasmin (or any variant or derivative thereof or alternative therefore) according to the invention may, depending on its ultimate use and mode of administration, comprise one or more further active ingredients such as an anticoagulant, a further thrombolytic agent, an anti-inflammatory agent, an antiviral agent, an antibacterial agent, an antifungal agent, an anti-angiogenic agent, an anti-mitotic agent, an antihistamine or anesthetic.

"Anticoagulants" include hirudins, heparins, coumarins, low-molecular weight heparin, thrombin inhibitors, platelet inhibitors, platelet aggregation inhibitors, coagulation factor inhibitors, anti-fibrin antibodies and factor VHI-inhibitors (such as those described in WO 01/04269 and WO 2005/016455).

"Thrombolytic agents" include wild-type plasmin, wild-type plasminogen, urokinase, streptokinase, tissue-type plasminogen activator (tPA or alteplase), urokinase-type plasminogen activator (uPA) and staphylokinase or any variant or derivative of any thereof such as APSAC (anisoylated plasminogen streptokinase activator complex), reteplase, tenecteplase, scuPA (single chain uPA), or a combination of any thereof.
"Anti-inflammatory agents" include steroids (e.g. prednisolone, methylprednisolone, cortisone, hydrocortisone, prednisone, triamcinolone, dexamethasone) and non-steroidal anti-inflammatory agents (NSAIDs; e.g. acetaminophen, ibuprofen, aspirin).

"Antiviral agents" include trifluridine, vidarabine, acyclovir, valacyclovir, famciclovir, and doxuridine.

"Antibacterial agents" or antibiotics include ampicillin, penicillin, tetracycline, oxytetracycline, framycetin, gatifloxacin, gentamicin, tobramycin, bacitracin, neomycin and polymyxin.

"Anti-mycotic/fungistatic/antifungal agents" include fluconazole, amphotericin, clotrimazole, econazole, itraconazole, miconazole, 5-fluorocytosine, ketoconazole and natamycin.

"Anti-angiogenic agents" include antibodies (or fragments thereof) such as anti-VEGF (vascular endothelial growth factor) or anti-PIGF (placental growth factor) antibodies and agents such as macugen (pegaptanib sodium), tryptophanyl-tRNA synthetase (TrpRS), anecortave acetate, combrestatin A4 prodrug, AdPEDF (adenovector capable of expressing pigment epithelium-derived factor), VEGF-trap, inhibitor of VEGF receptor-2, inhibitors of VEGF, P1GF or TGF-β, Sirolimus (rapamycin) and endostatin.

"Anti-mitotic agents" include mitomycin C and 5-fluorouracil.

"Antihistamine" includes ketotifen fumarate and pheniramine maleate.

"Anesthetics" include benzocaine, butamben, dibuacaine, lidocaine, oxybuprocaine, pramoxine, proparacaine, proxymetacaine, tetracaine and amethocaine.

"Contacting", when used herein, means any mode of administration that results in interaction between a composition such as a medicament and the tissue, body fluid, organ, organism, etc. with which said composition is contacted. The interaction between the composition and the tissue, body fluid, organ, organism, etc can occur starting immediately or nearly immediately with the administration of the composition, can occur over an extended time period (starting
immediately or nearly immediately with the administration of the composition), or can be delayed relative to the time of administration of the composition.

Any method of contacting a pathological fibrin deposit that provides (either immediately, delayed or over an extended time period) an effective amount of a plasmin (or any variant or derivative thereof or alternative therefore) to such fibrin deposit can be utilized. If such fibrin deposit is associated with a blood clot, the plasmin (or any variant or derivative thereof or alternative therefore) can be delivered intra-arterially, intravenously, or locally (within short distance of the clot or even in the clot) by means of injection and/or infusion and/or a catheter.

When using plasmin (or any variant or derivative thereof or alternative therefore) in enzymatic debridement, it may be included in a gel-like composition capable of being applied topically, or may be applied in liquid form.

Any method of contacting the eye vitreous and/or aqueous humor that provides (either immediately, delayed or over an extended time period) an effective amount of a plasmin (or any variant or derivative thereof or alternative therefore) to the vitreous and/or aqueous humor can be utilized. One method of contacting the vitreous and/or aqueous humor is by one or more intraocular injections directly into the vitreous and/or aqueous humor. Alternatively, said contacting may involve subconjunctival, intramuscular or intravenous injections. A further alternative contacting method involves placing an intra-vitreal implantable device such as OCUSERT® (Alza Corp., Palo Alto, California) or VITRASERT® (Bausch & Lomb Inc., Rochester, New York). Contacting the vitreous and/or aqueous humor with an effective amount of a plasmin (or any variant or derivative thereof or alternative therefore) may be in a continuous fashion using a depot, sustained release formulation or any implantable device suitable thereto.

The term "effective amount" refers to the dosing regimen of the medicament according to the invention, in particular of the active ingredient of the medicament according to the invention, i.e., plasmin or an active truncated variant thereof (or any alternative therefore as described above). The effective amount will generally depend on and will need adjustment to the mode of contacting or administration and the condition to be treated. The effective amount of the
medicament, more particular its active ingredient, is the amount required to obtain the desired clinical outcome or therapeutic or prophylactic effect without causing significant or unnecessary toxic effects. To obtain or maintain the effective amount, the medicament may be administered as a single dose or in multiple doses. The effective amount may further vary depending on the severity of the condition that needs to be treated or the expected severity of the condition that needs to be prevented; this may depend on the overall health and physical condition of the patient and usually the treating doctor's or physician's assessment will be required to establish what is the effective amount. The effective amount may further be obtained by a combination of different types of administration. The medicament may be administered as a solution (liquid or semi-liquid, e.g., gel-like or in dispersion or suspension, colloidal, in emulsion, nanoparticle suspension) or as a solid (e.g. tablet, minitablet, hard- or soft-shelled capsules).

For purposes of thrombolysis, plasmin dosage and duration of plasmin therapy will typically depend on the size and location of the blood clot as well as on the size, weight and age of the patient. If a clot is venous, treatment with plasmin may continue for days whereas only hours of plasmin therapy may be required if the clot is arterial. A myocardial infarction may be treated with a short single dose treatment whereas conditions such as thrombophlebitis and pulmonary embolism may require longer multiple dose treatment. Prolonged continuous and/or intermittent thrombolytic plasmin therapy may be applied to treat a coronary occlusion or in case of prophylactic therapy in order to reduce the risk of clot formation in subjects known to have an increased risk to develop clot formation. A further factor influencing plasmin dosage includes the circulating levels plasmin inhibitors such as a2-antiplasmin and/or a2-macroglobulin, the initial level of which being patient-dependent. It may be advisable to adjust the plasmin dosage such that no more than 15% of the total circulating a2-antiplasmin is remaining in order to achieve efficient thrombolytic therapy. For the purpose of inducing thrombolysis, a contacting method delivering a plasmin or any variant or derivative thereof or alternative therefore at a short distance proximal to a thrombus may be advantageous as the exposure to serum inhibitors is reduced. Such contacting method typically involves delivery via a catheter device. For use in thrombolysis, typical plasmin dosages range from 500 microgram/body weight to 10 milligram/kg body weight given as a single bolus or divided over 1 initial bolus injection followed by 1 or more repeat bolus injections. Plasmin may alternatively be administered over an extended time
period, e.g. by infusion or by drug delivery micropump. Plasmin dosages for continued administration may range from 1 to 10 mg/kg/hour.

A typical plasmin dosage for inducing posterior vitreous detachment, vitreous liquefaction, clearance of vitreal blood or hemorrhages, or clearance of toxic materials or foreign substances from the vitreous cavity may be in the range of about 0.1 microgram to about 250 microgram per eye per dose, which can be delivered in a diluent or carrier volume of about 50 microliter to about 300 microliter per eye per dose. The diluent or carrier may e.g. be a sterile Balanced Salt Solution (BSS or BSS Plus), a physiologic saline solution or a solution containing 1-10 mM citric acid. In one embodiment plasmin is delivered to the eye in a dose of 125 microgram contained in 0.1 mL diluent or carrier. In the case of planned surgical vitrectomy, said plasmin may be delivered to the eye 15 to 300 minutes, or 15 to 120 minutes prior to the vitrectomy. Alternatively, the purpose of administering plasmin in the eye is to avoid surgical vitrectomy, or to facilitate subsequent surgical vitrectomy in case plasmin treatment itself would not be able to achieve full posterior vitreous detachment. When using plasminogen as an alternative source for plasmin (see "plasmin" definition), up to 250 microgram of plasminogen can be introduced per eye and said plasminogen may be accompanied by up to 2000 IU of urokinase or streptokinase as plasminogen activator or by up to 25 microgram of tPA. When used in the eye, plasmin or plasminogen administration may further be accompanied by administration of a gaseous adjuvant such as air, an expanding gas or liquefiable gas, or mixtures thereof, as long as it is non-toxic to the eye. Other suitable gaseous materials include SF6 (sulfur hexafluoride) and perfluorocarbons, such as C2F6 (hexafluoroethane), C3Fs (octafluoropropane), C4Fs (octafluorocyclobutane), oxygen, nitrogen, carbon dioxide, argon, and other inert gases. The volume of the gaseous material that is introduced into the eye can vary depending on the gaseous material, the patient, and the desired result. For example, the volume of air that is injected into the posterior chamber can range from about 0.5 mL to about 0.9 mL. Other gaseous materials, such as SF6 and perfluorocarbon gases can range from about 0.3 mL to 0.5 mL. Preferably, the gaseous material is introduced into the posterior chamber of the eye in an amount sufficient to compress the vitreous against the posterior hyaloid and form a cavity in the vitreous without damaging the eye.

In preferred embodiments, the gaseous adjuvant is introduced into the vitreous to form a cavity that fills about 40% to about 60% of the internal volume of the intraocular cavity.
The above recited dosages are indicative values not meant to be limiting in any way. Said dosages furthermore refer to wild-type plasmin or plasminogen or any active or activatable truncated variant thereof. When using a plasmin with increased stability according to the invention (or any variant or derivative thereof or alternative therefore), and depending on the ultimate stability and residual activity of a plasmin according to the invention, dosages may be similar, higher or lower to obtain the same or better overall clinical effect as obtained with wild-type plasmin. Dosage of a plasmin according to the invention may also depend on the rate of inhibition by endogenous inhibitors such as a2-antiplasmin.

The invention further relates to methods for screening for autoproteolytically stable plasmin variants, said methods comprising:

(i) providing a plasmin variant according to the invention, and providing wild-type plasmin,
(ii) comparing the autoproteolytic stability of the variant plasmin and wild-type plasmin provided in (i), and
(iii) selecting from (ii) a variant which retains proteolytic activity, and of which autoproteolytic stability is increased relative to the autoproteolytic stability of wild-type plasmin.

The above screening methods may further comprise a step wherein the proteolytic activity of the autoproteolytically stable plasmin variant is determined.

Many products including medicines (here to be understood specifically as user-ready active ingredient, i.e. in the final formulation for administration to a patient) and bulk-stored active ingredients of medicines are usually stored for a considerable amount of time prior to use. It is of interest to extend the shelf-life of products as long as possible. With the shelf-life is meant the time during which the product can be used safely and during which the product retains it potent utility, i.e. its activity in the case of a medicine and/or its active ingredient. Typically, the shelf-life is indicated on a product or its package. Once the shelf-life has expired, the safe and potent utility of a product is no longer guaranteed. A further important aspect in storing products is the storage temperature at which the desired shelf-life can be achieved. For example, the shelf-life of a product stored at +4°C or average refrigerator temperature may amount to 12 months whereas
the shelf-life of the same product stored at -20°C or average freezer temperature may amount to 36 months. Logistically, however, maintaining a cold chain at freezing temperatures, e.g. -20°C, is much more complex, difficult and expensive than maintaining a cold chain at +4°C. Thus, it may still be attractive to have a shorter, but sufficiently long shelf-life combined with the possibility to store a product at +4°C. The present invention offers a solution for extending, enhancing or increasing the shelf-life or long-term storage stability of plasmin or any active fragment or derivative thereof or of a composition comprising plasmin or any active derivative thereof. The solution resides in making available plasmin variants as herein described, said variants having an enhanced stability, which, intrinsically, increases, enhances or extends their shelf-life.

The invention likewise relates to methods for enhancing long-term storage stability of a plasmin-comprising composition, said methods comprising the step of identifying an autoproteolytically stable plasmin variant capable of being stored over a long time without significant loss of proteolytic activity. For determining long-term stability, a plasmin preparation according to the invention is aliquoted and activity measurements are performed repeatedly during the envisaged storage term. If the envisaged storage term is, e.g., 24 months, activity measurements can be performed, e.g. every month. The allowable loss of proteolytic activity at the end of the envisaged storage term will largely depend on the envisaged clinical application but typically may be no more than e.g. 10% to 15%.

The invention further relates to methods for producing a plasminogen variant according to the invention, said method including the steps of:

(i) introducing a nucleic acid encoding a plasminogen according to the invention in a suitable host cell capable of expressing said plasminogen;

(ii) growing the host cell obtained in (i) under conditions and during a time sufficient for expression of said plasminogen in said host cell; and

(iii) harvesting the plasminogen expressed in (ii).

The invention further relates to methods for producing a plasmin variant according to the invention, said method including the steps of:
(i) introducing a nucleic acid encoding a plasminogen according to the invention in a suitable host cell capable of expressing said plasminogen;
(ii) growing the host cell obtained in (i) under conditions and during a time sufficient for expression of said plasminogen in said host cell;
(iii) harvesting the plasminogen expressed in (ii);
(iv) activating the plasminogen of (iii) to plasmin.

Suitable host cells and methods for expression and production are disclosed in e.g. WO 90/13640 (insect cells), WO 2002/050290 and WO 03/066842 (yeast cells), WO 2008/054592 (bacterial cells/refolding process) and WO 2005/078109 (duckweed transgenic plants or transgenic plant cells).

The invention also relates to (an) isolated nucleic acid sequence(s) encoding a plasminogen variant or plasmin variant according to the invention. The invention also relates to (a) recombinant vector(s) comprising such nucleic acid. The invention also relates to (a) host cell(s) transformed with such nucleic acid or with such recombinant vector.
EXAMPLES

EXAMPLE 1. Construction and expression of plasminogen variants, and activation to plasmin.

5 Expression vector
The pPICZaA secretion vector purchased from Invitrogen Corporation (Carlsbad, California) was used to direct expression and secretion of recombinant human microplasminogen in Pichia pastoris.

10 This vector contains the secretion signal of the Saccharomyces cerevisiae a-factor prepropeptide. A Xhol recognition sequence is present at the COOH-terminus of the a-factor secretion signal, immediately upstream of the Lys-Arg site that is cleaved by Kex2 to remove the secretion signal from the mature protein. This Xhol restriction site may be used to clone the gene of interest flush with the Kex2 cleavage site by synthesizing the gene with the Xhol and Kex2 recognition sites at its the 5’ end. The recombinant gene of interest will then be expressed with the native NH$_2$-terminus. Engineered immediately downstream from the a-factor secretion signal in the pPICZaA vector is a multiple cloning site with recognition sites for the restriction enzymes EcoRI, Sfil, Kpnl, SacII and Xbal to facilitate the cloning of heterologous genes.

20 Gene synthesis
To improve expression of human microplasminogen in Pichia pastoris, genes encoding the human microplasminogen and variants thereof were synthesized de novo taking into account the preferred codon usage by Pichia pastoris.

25 To design the codon-optimized gene sequence, the human microplasminogen amino acid sequence (SEQ ID NO:19) was imported in the program Gene Designer which is developed by DNA2.0 (Menlo Park, California) and is freely available on the internet. This sequence was backtranslated into DNA sequence using the Pichia pastoris codon usage table provided with the program. The nucleotide sequence was then checked manually and adjusted to better fit Escherichia coli codon usage (SEQ ID NO:20). In addition, 6-base pair palindromic sequences
and nucleotide repetitions were removed when possible. At the 5' end, an XhoI restriction site and the Kex2 cleavage site were added and at the 3' end, an XbaI restriction site was added.

Mutations were introduced by site-directed mutagenesis using the QuikChange II Site Directed Mutagenesis Kit from Agilent (La Jolla, California) in the wild-type microplasminogen sequence or in variant microplasminogen sequences in which specific other amino acid(s) were already changed. The E. coli strain TOP10 (Invitrogen) was transformed with the site-directed mutagenesis mixture and ampicillin resistant clones were selected. Sequence determination of the resulting plasmid clones confirmed the precise mutagenesis of the targeted microplasminogen coding region, as well as the absence of unwanted mutations in the coding region.

The following primers were used for site-directed mutagenesis:

**Vail Ile mutation**

GAAGTGTCAGGTCGT^TTGTCGGTGGCTGTGGCTC (sense; SEQ ID NO:21) and GAGCCACACAGCCACCGACA4JACGACCTGGACACTTC (antisense; SEQ ID NO:22)

In a first variant, the valine at position 1 is substituted by an isoleucine. Vail is encoded by the codon GTT at positions 58-60. The nucleotides GTT (positions 58-60) were changed into ATT, changing Vail into Ile in the microplasminogen protein (nucleotide sequence is in SEQ ID NO:23 and the deduced amino acid sequence in SEQ ID NO:24).

**Expression of microplasminogen variants and activation to plasmin**

The microplasminogen variants and activated microplasmin variants are obtained by following essentially the procedure as outlined in Example 2 of WO 02/50290.

Prior to activation, the microplasminogen mutants were purified by immuno-affinity directly from the Pichia pastoris supernatants. A murine anti-human microplasmin antibody (raised in Balb/c mice using microplasmin as antigen; produced by hybridoma cell line 5D10A4, available at ThromboGenics N.V.) was coupled on sepharose beads according to the protocol no.
71500015AD from GE Healthcare. Following this protocol, 7.5 mL of immuno-affinity resin were prepared from 45 mg of antibody and packed in a XK 16/20 column. Crude supernatant 200-400 mL (0.2 μ filtered from Pichia culture/ pH 6.0) was directly loaded on the 5D10A4 affinity column. After a wash step (100 mM KH2PO4, 0.5M NaCl, pH 6.2, 10 column volumes), the microplasminogen variant was eluted with a 0.2M Glycine-HCl, pH 3.0 buffer. The eluate (fractions 4-6) was neutralized and dialyzed against 25mM Sodium Phosphate buffer, pH 7.2).

Amino acid sequences and nucleotide sequences of the above described wild-type and variant microplasminogen species are listed hereafter.

SEQ ID NO:19 - Wild-type Human microplasminogen amino acid sequence
APSFDCGKPQVEPKKCPGRWGGCVAHPHHSWPQVSLRTRFGMHFCGGTLLISPEWVLT
AAHCLEKSPRPSSYKVLGHAQWELEPHVQEIEVSRLFLEPRKDIALLKLSSPAVRDK
VIPACLPSPNYVVAARDECITFGWGETQFGAGGLKEAQLPVIENKVCNRYEFNLGNR
QSTELCAGHLAGGDSCQGDSGGLVCFEKDKJLYLQVTGVTSLGCARPKNPGVYVRVS
RFVTWIEGVMRNN

SEQ ID NO:20 - Artificial nucleic acid sequence with optimized codon usage for expression in Pichia. The nucleic sequence encodes the wild-type human microplasminogen amino acid sequence of SEQ ID NO:19
GCACCTTCCATCGACTGTGTAAGGCTCATCAGTGTCACACCTAAGGAGGTCCAGGTCGT
GTTGTCGTTGGCTGT GTGGCTCAT CTCATTTCTGGCCTTGGGC AAGTGTCCTTT AGAA
CTAGATTGCTGATCGACTCTCCTCTTGTTGGCCTGACCTTGATCTACCTGAAAGGGTCTTAAC
CGCAGCTCATGTCTGGAGAACTCCACCGTCCATCTTCAATAAGGTCATCCTTGGG
CGCACTACAGGATGCAATCTTGGCTGCTCGTGAGGAGATCGAAGTCCTCTCAGGTT
GGTTCTGGAACAGGACATTGCTCGTCTCTGTGAGGTCTGCACTCCTCTCGCTGGT
ATTACGCAAGGTAACCTGGGCTGCCTGAGGCTGCTGTCCTCCTCGGG
GTACCCGATTGCGTCATCTGTGCTGAGGACTCAAGGTTCCTGTCGTTCCGACC
ATTGCCAAAGGTCATCTGGGCTGAGGACTCAAGGTTCCTGTCGTTCCGACC
TTCCATTGGGAGACAAGGACTCTTTGCTGAGGACTCAAGGTTCCTGTCGTTCCGACC
ACTGATAGTTTGTCAAGGATCTCTGGGAGACAAGGACTCTTTGCTGAGGACTCAAGGTTCCTGTCGTTCCGACC
TACATCTTACAAGGTGTTACGTCTTGGGGTCTTGGATGTGCTCGTCCTAACAAGCCA
GGTGCTCTACGTCAGGCTCCAGATTCGAACCTTGAGATCGAAGGTGTCATGCGTAAC
AACTAA

SEQ ID NO:23 - Microplasminogen variant with the Vallle substitution (mutated codon in bold italics underlined)
GCACCTTCATTGCAACTGTGGTAAAGCCTCAGGTCGAACCTAAGAAGTGTCCAGGTGT
47TGTCAGTGCTGTGTGCTCATCCTACATTCTTGCCATTGCAAGGTGTCTCTTAGAAA
CTAGATTTGGTATGCACTTCTGTGTTGGGACCTTGTATCTACGTGTAAC
AACTAA

SEQ ID NO:24 - Deduced amino acid sequence of SEQ ID NO:23 (the introduced amino acid mutation is indicated in bold/italic and is underlined)
APSFDCGKPQVEPKKCPGR/VGGCVAHPHSWPWQVSLRTRFGHCFGGTLISPEWVL
AAHCLEKSPRPSSYKVILGAHQEVNLEPHVQEIEVSRLFLEPTRKDIALLKLSSPAVITDK
VIPACLPSNPYWADRTSCEFIFWGETQGTFGAGLLKEAQLPVIENKVCNRYEFLNGRV
QSTELCAGHLAGGTDQGDSGPVLCFEDKYILQGVTSGWGLGCARPKNPGVYVVRVSRFVTWIEGVMRNN

Results:
The kcat and Km values obtained for various microplasmin mutants are listed in Table 1 below.
Table 1.

<table>
<thead>
<tr>
<th>Variant</th>
<th>Kinetic parameters $K_{cat}/K_m$ (M$^{-1}$s$^{-1}$)</th>
<th>Autolysis rate constant $k$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>$6.9 \times 10^7$</td>
<td>123</td>
</tr>
<tr>
<td>V11</td>
<td>$1.0 \times 10^6$</td>
<td>33</td>
</tr>
<tr>
<td>V11 K147E</td>
<td>$6.7 \times 10^5$</td>
<td>7</td>
</tr>
</tbody>
</table>

**EXAMPLE 2. Therapeutic efficacy of plasmin variants in in vitro or in vivo models.**

2.1 Effect of plasmin variants on cerebral infarct size.

The efficacy of the plasmin variants of the invention in reducing cerebral infarct size can be performed in a murine cerebral infarct model such as described in Example 2 of WO 00/18436, or according to Welsh et al. (1987, J Neurochem 49, 846-851). The beneficial effect of wild-type plasmin on cerebral infarct size was demonstrated in Example 5 of WO 00/18436. A similar experiment is performed with any of the plasmin variants of the invention and the beneficial effect of these plasmin variants is measured and compared to the beneficial effect of wild-type plasmin.

2.2 In vivo thrombolytic activity of plasmin variants.

The rabbit extracorporeal loop thrombolysis model (Example 6 of WO 02/50290; Hotchkiss et al., 1987, Thromb Haemost 58, 107 - Abstract 377), the dog circumflex coronary artery copper coil-induced thrombosis model (Example 8 of WO 02/50290; Bergmann et al., 1983, Science 220, 1181-1183) or the rabbit jugular vein thrombosis model (Collen et al., 1983, J Clin Invest 71, 368-376) can be used to demonstrate in vivo thrombolytic activity of the plasmin variants of the invention. The beneficial effect of wild-type plasmin on thrombolysis was demonstrated with these models as described in Examples 7 and 9 of WO 00/18436 and by Collen et al. (1983). Similar experiments are performed with any of the plasmin variants of the invention and the beneficial effect of these plasmin variants is measured and compared to the beneficial effect of wild-type plasmin.

2.3 In vitro thrombolytic activity of plasmin variants.
An in vitro model of peripheral arterial occlusion (PAO) is described in Example 6 of WO 01/36609 and the thrombolytic efficacy of wild-type plasmin was demonstrated in this model. A similar experiment is performed with any of the plasmin variants of the invention and the beneficial effect of these plasmin variants on thrombolysis of peripheral arterial occlusions is measured and compared to the beneficial effect of wild-type plasmin.

2.4 Liquefaction of eye vitreous and posterior vitreous detachment induced by plasmin variants

Example 5 of WO 2004/052228 discloses an assay for determining the efficacy, as well as the efficacy of microplasmin in liquefying the vitreous in post-mortem pig eyes. Example 6 of WO 2004/052228 discloses an assay for determining the efficacy, as well as the efficacy of microplasmin in inducing posterior vitreous detachment (PVD) in human post-mortem eyes. Induction of vitreous liquefaction and PVD by the plasmin variants of the invention is demonstrated in similar post-mortem models.

2.5 In vivo PVD induced by plasmin variants

Example 7 of WO 2004/052228 discloses an assay for determining the efficacy, as well as the efficacy of microplasmin in inducing PVD in an in vivo feline model. Induction of PVD by the plasmin variants of the invention is demonstrated in a similar in vivo model.

Intravitreal injection of the plasmin variant Vallle (VII)

The effect of VII microplasmin variant on posterior vitreal detachment (PVD) was examined after intravitreal injection. Briefly, adult C57BL/6 mice were anesthetized with Nembutal (0.6mg/kg body weight). Intravitreal injections were done using an intraocular injection kit, a 10µl syringe with 35G beveled needle, and a micro-pump injection apparatus. Each injection of one µl of vehicle containing either wild-type microplasmin or the VII microplasmin variant at various concentrations was performed under a dissection microscope upon depression of a foot switch. The needle tip was passed through the sclera posterior to the limbus and was positioned to avoid touching the lens. Depression of the foot switch caused the jet of injected product into the midvitreous cavity.

Five days post injection the mice were killed and their eyes were fixed in 1% paraformaldehyde. The eyes were paraffin wax embedded followed by sectioning, and sections were stained witht
the period acid-Schiff (PAS) reagent. To assess induction of PVD, morphological analysis was performed on the entire eye's sections using light microscopy.

**Effect of intravitreal injection of microplasmin and VII**

Representative light microscopy image of VII injected eyes at 5 days post injections is shown in Figure 3. PAS stained eye globes sections displayed detachment of the vitreous from the retinal surface following injection of VII. Microplasmin injected animals demonstrated a 20% induction of PVD as compared to 50% induction of PVD after VII injection (Table 2). This apparent 2 to 3 times increase in PVD induction was observed at all the concentrations tested.

**Table 2. PVD induction by VII and wild-type microplasmin in mouse eyes.**

<table>
<thead>
<tr>
<th>concentration</th>
<th>incubation time</th>
<th>wild-type microplasmin</th>
<th>VII mutant microplasmin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% PVD</td>
<td>#</td>
<td>% PVD</td>
</tr>
<tr>
<td>60 ng</td>
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<td>0%</td>
<td>(0/4)</td>
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<tr>
<td></td>
<td>10 days</td>
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<tr>
<td>30 ng</td>
<td>5 days</td>
<td>20%</td>
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<td>0%</td>
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<td>20%</td>
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<tr>
<td></td>
<td>30 days</td>
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<td>(0/4)</td>
</tr>
</tbody>
</table>
CLAIMS

1. An isolated plasminogen variant or plasmin variant obtainable therefrom, or an isolated plasmin variant, or a proteolytically active or reversibly inactive derivative of any of said plasmin variant, wherein said variants are comprising an activation site and a catalytic domain, characterized in that said catalytic domain contains a mutation of one or more amino acids at positions 1 to 4 of the human plasmin catalytic domain or at positions corresponding thereto in a non-human plasmin catalytic domain, wherein said human plasmin catalytic domain is starting with the amino acid valine at position 1 which is the same valine amino acid occurring at position 562 of human Glu-plasminogen.

2. The plasminogen variant, plasmin variant, or plasmin derivative according to claim 1 wherein, if said catalytic domain is mutated at position 1, (i) the amino acid at position -1 relative to the plasmin catalytic domain is an arginine, lysine or other amino acid that maintains functionality of the activation site, (ii) the amino acid at position 24 of the human plasmin catalytic domain, or at the corresponding position of a non-human plasmin catalytic domain, is a methionine, and (iii) the amino acid at position 1 is mutated into an amino acid different from glycine or proline.

3. The plasminogen variant, plasmin variant, or plasmin derivative according to claim 1 wherein, if said catalytic domain is mutated at positions 1 and 2, the amino acid at position 24 of the human plasmin catalytic domain, or at the corresponding position of a non-human plasmin catalytic domain, is a methionine.

4. The plasminogen variant, plasmin variant, or plasmin derivative according to any of claims 1 to 3 wherein said mutation reduces the extent of autoproteolytic degradation of said plasmin variant compared to the extent of autoproteolytic degradation of wild-type plasmin, such as determined with a chromogenic or biological substrate activity assay.
5. The plasminogen variant, plasmin variant, or plasmin derivative according to any of claims 1 to 4 wherein said mutation is the mutation of the amino acid valine at position 1 of the catalytic domain into isoleucine.

6. The plasmin variant or plasmin derivative according to any of claims 1 to 5 further characterized in that it is its autolysis constant is at most 95% of the autolysis constant of wildtype plasmin.

7. The plasmin variant or plasmin derivative according to any of claims 1 to 5 further characterized in that the catalytic constant $k_{cat}$ is in the range of 10% to 200% of the $k_{cat}$ of wildtype plasmin.

8. The plasmin variant or plasmin derivative according to any of claims 1 to 5 further characterized in that its autolysis constant is at most 95% of the autolysis constant of wildtype plasmin and its catalytic constant $k_{cat}$ is in the range of 10% to 200% of the $k_{cat}$ of wildtype plasmin.

9. The isolated plasminogen variant, plasmin variant, or plasmin derivative according to any of claims 1 to 8 wherein said plasminogen or plasmin is Glu-plasminogen or Glu-plasmin, Lys-plasminogen or Lys-plasmin, midiplasminogen or midiplasmin, miniplasminogen or miniplasmin, microplasminogen or microplasmin, deltaplasminogen or deltaplasmin.

10. The isolated plasminogen variant, plasmin variant, or plasmin derivative according to any one of claims 1 to 9, or a combination of any thereof for use as a medicament.

11. A composition comprising the isolated plasminogen variant, plasmin variant, or plasmin derivative according to any one of claims 1 to 9, or a combination of any thereof, and at least one of a pharmaceutically acceptable diluent, carrier or adjuvant.

12. The composition according to claim 11 further comprising at least one of an anticoagulant, a thrombolytic agent, an anti-inflammatory agent, an antiviral agent, an antibacterial agent, an
antifungal agent, an anti-angiogenic agent, an anti-mitotic agent, an antihistamine or an anaesthetic.

13. A method for screening for an autoproteolytically stable plasmin variant, said method comprising:
   (i) providing a plasmin variant according to any of claims 1 to 5, and providing wild-type plasmin,
   (ii) comparing the autoproteolytic stability of the variant plasmin and wild-type plasmin provided in (i), and
   (iii) selecting from (ii) a variant which retains proteolytic activity, and of which autoproteolytic stability is increased relative to the autoproteolytic stability of wild-type plasmin.

14. A method for producing a plasminogen variant according to any of claims 1 to 9, said method including the steps of:
   (i) introducing a nucleic acid encoding a plasminogen according to any of claims 1 to 9 in a suitable host cell capable of expressing said plasminogen;
   (ii) growing the host cell obtained in (i) under conditions and during a time sufficient for expression of said plasminogen in said host cell; and
   (iii) harvesting the plasminogen expressed in (ii).

15. A method for producing a plasmin variant according to any of claims 1 to 9, said method including the steps of:
   (i) introducing a nucleic acid encoding a plasminogen according to any of claims 1 to 9 in a suitable host cell capable of expressing said plasminogen;
   (ii) growing the host cell obtained in (i) under conditions and during a time sufficient for expression of said plasminogen in said host cell;
   (iii) harvesting the plasminogen expressed in (ii);
   (iv) activating the plasminogen of (iii) to plasmin.
16. An isolated nucleic acid sequence encoding the plasminogen variant or plasmin variant according to any one of claims 1 to 9.

17. A recombinant vector comprising the nucleic acid according to claim 16.

18. A host cell transformed with the nucleic acid according to claim 16 or the vector according to claim 16.
<table>
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<th>Genbank Accession</th>
<th>Description</th>
<th>SEQ ID No.</th>
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</thead>
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<tr>
<td>1</td>
<td>Homo sapiens</td>
<td>Genbank AAA36451</td>
<td>human</td>
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</tr>
<tr>
<td>2</td>
<td>Canis familiaris</td>
<td>Genbank XP_533468</td>
<td>dog</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>Pan troglodytes</td>
<td>Genbank XP_001152889</td>
<td>chimpanzee/ isoform 3</td>
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</tr>
<tr>
<td>4</td>
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<td>chimpanzee/ isoform 2</td>
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**FIGURE 2/12**
INTERNATIONAL SEARCH REPORT

PCT/EP2012/065832

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K38/48 C12N9/64
ADD. C07K14/47 C12N9/68 C12N15/11

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)
A61K C12N

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 practicable, search terms used)

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category Citation of document, with indication, where appropriate, of the relevant passages Relevant to claim No.


A WO 2011/004011 Al (THROMB0GENICS NV [BE]); ZWAAL RICHARD REINER [BE]) cited in the application on the whole document 1-18

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents :
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" later document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referred to in oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"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
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

Date of the actual completion of the international search: 6 November 2012

Date of mailing of the international search report: 19/11/2012

Name and mailing address of the ISA:
European Patent Office, P.B. 5018 Patentlaan 2
NL-2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer:
Seranski, Peter

Form PCT/ISA/210 (second sheet) (April 2005)
INTERNATIONAL SEARCH REPORT

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. □ Claims Nos.:  
   because they relate to subject matter not required to be searched by this Authority, namely:

2. X claims NOS.: 1-18 (partially) because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
   see FURTHER INFORMATION sheet PCT/ISA/210

3. □ Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. □ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. □ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. □ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. □ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

□ The additional search fees were accompanied by the applicant’s protest and, where applicable, the payment of a protest fee.

□ The additional search fees were accompanied by the applicant’s protest but the applicable protest fee was not paid within the time limit specified in the invitation.

□ No protest accompanied the payment of additional search fees.
Conti nuati on of Box 11.2

Claims Nos.: 1-18(par ti al ly)

Present claims 1-18 relate to an extremely large number of possible proteins. Support and disclosure in the sense of Article 6 and 5 PCT is to be found however for only a very small proportion of the proteins claimed. The claimed plasminogen variants are not clearly defined because they contain at least 4 wildtype-substituents. This would require an equally unquantifiable and thus unreasonable amount of experimentation, imposing a severe and undue burden on all those wishing to ascertain the scope of the claim, which conflicts with the clarity requirement of Article 6 PCT. Non-compliance with the substantive provisions is such that a full search of the whole claimed subject-matter of the claim was not carried out (PCT Guidelines 9.19 and 9.23). The extent of the search was consequently limited to the clearly defined examples in the description and a general satisfaction of their structural formulae, i.e. Seq-ID-24.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guidelines C-VI, 8.2), should the problems which led to the Article 17(2) declaration be overcome.
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