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Rekombinánsan módosított plazmin

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(54) **RECOMBINANTLY MODIFIED PLASMIN**
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- **LIN L-F ET AL: "Epsilon amino caproic acid inhibits streptokinase-plasminogen activator complex formation and substrate binding through kringle-dependent mechanisms" BIOCHEMISTRY, AMERICAN CHEMICAL SOCIETY, EASTON, PA.; US, vol. 39, no. 16, 25 April 2000 (2000-04-25), pages 4740-4745, XP002348009 ISSN: 0006-2960**

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Description**Background of the Invention**

[0001] Human plasminogen is a single-chain protein containing 791 amino acid residues. Activation of plasminogen to plasmin results from a single cleavage of the Arg561-Val562 peptide bond in the zymogen. The resulting plasmin molecule is a two-chain, disulfide-linked serine protease with trypsin-like specificity (cleaves after Lys and Arg).

[0002] The amino-terminal heavy chain of plasmin (residues 1-561, ~60 kDa) is composed of five kringle domains, each containing approximately 80 amino acid residues. The kringle domains are responsible for the regulatory properties of plasminogen, such as interaction with activation inhibitors, e.g., Cl^{-1} ions; with activation stimulators, e.g., ϵ -aminocaproic acid; with mammalian and bacterial cells; and with other proteins, such as the plasmin physiological substrate, fibrin and plasmin inhibitor, α 2-antiplasmin. Of all five kringles, kringle 1 is one of the most multi-functional: its lysine-binding activity has been shown to be responsible for plasmin interaction with α 2-antiplasmin and fibrin. See Wiman, B., et al., Biochim. Biophys. Acta 579: 142-154 (1979); and Lucas, M.A., et al., J. Biol. Chem. 258: 4249-4256 (1983).

[0003] The C-terminal light chain of plasmin (residues 562-791, ~25kDa) is a typical serine protease, homologous to trypsin and containing the classic serine protease catalytic triad: His603, Asp646 and Ser741. Plasminogen contains 24 disulfide bridges and 2 glycosylation sites on Asn289 and Thr346.

[0004] The limited proteolysis of plasminogen by elastase has been shown to result in three major fragments (Sottrup-Jensen, L., et al., Prog. Chem. Fibrinol. Thrombol. 3: 191-209 (1978)). First fragment, K1-3, includes the first three kringles and can be isolated in two versions, Tyr80-Val338 and Tyr80-Val354. The second fragment, K4, corresponds to the fourth kringle and includes residues Val355-Ala440. The last, C-terminal fragment (the so-called mini-plasminogen) includes residues Val443-Asn791 and consists of the fifth kringle and the serine protease domain. Mini-plasminogen can be activated in the same way as plasminogen, forming mini-plasmin.

[0005] Because of the complex structure of the full-length plasminogen molecule, bacterial expression systems have not proven useful for recombinant plasminogen production. Plasminogen is produced in the form of insoluble inclusion bodies and is not re-foldable from that state. Further, the expression of plasminogen in mammalian cells is complicated by intracellular activation of plasminogen into plasmin and the resulting cytotoxicity. Production of fully active plasminogen using insect cells is possible, however, this system is not suitable for large-scale production due to low yield. Further, as with any recombinant protein scheme, the potential exists for encountering immunogenicity problems in the subject receiving the therapeutic recombinant protein.

[0006] Immunogenicity can be a barrier to the effective and/or efficient utilization of certain recombinant protein therapeutic schemes. Immunogenicity is a complex series of responses to a substance (e.g., the chemical structure of a protein including the amino acid sequence) that is perceived as foreign and may include production of neutralizing and non-neutralizing antibodies, formation of immune complexes, complement activation, mast cell activation, inflammation, and anaphylaxis. Immunogenicity may limit the efficacy and safety of a protein therapeutic in multiple ways. Efficacy can be reduced directly by the formation of neutralizing antibodies. Efficacy may also be reduced indirectly, as binding to either neutralizing or non-neutralizing antibodies typically leads to rapid clearance from serum. Severe side effects and even death may occur when an immune reaction is raised. One special class of side effects results when neutralizing antibodies cross-react with an endogenous protein and block its function.

[0007] Accordingly, a modified recombinant protein, possessing the desirable characteristics (e.g., regions with native-like chemical structures) of plasmin/plasminogen while lacking certain negative characteristics and being capable of production in recombinant protein expression systems including bacterial cells in substantial quantities, is desirable.

[0008] The PCT Patent application WO 2007/047874 A disclosed delta-plasminogen, a deletion mutant plasminogen in which the middle portion of the molecule is removed, however the resulting molecule is different than those of the present invention.

[0009] On the other hand, Medynski D. et al (Medynski et al. "Refolding, purification, and activation of miniplasminogen and microplasminogen isolated from E. coli inclusion bodies", Protein Expression and Purification, Academic Press, San Diego, CA, vol. 52, no. 2, 26 October 2006, pages 395-402) disclose the refolding, purification, and activation of miniplasminogen isolated from E. coli inclusion bodies.

Summary of the Invention

[0010] In one aspect, the present invention provides a polynucleotide comprising a nucleotide sequence encoding a polypeptide having:

- (a) a single N-terminal kringle domain homologous to a kringle domain of native human plasminogen, wherein the last four amino acid residues within the kringle domain are V, P, Q, and C; and
- (b) a C-terminal domain activation site and serine protease domain homologous to the corresponding domains in

human plasminogen; wherein the polypeptide binds to immobilized lysine **and is capable of becoming activated to functional plasmin enzymes following an activation event that at least involves proteolytic cleavage of an Arg-Val peptide bond located between the kringle domain and the serine protease domain.**

[0011] In another aspect, the present invention provides a polypeptide **encoded by the above polynucleotide.**

[0012] In other aspects, the present invention provides an expression vector comprising **the** polynucleotide of the present invention. In one embodiment, the polynucleotide comprises a nucleotide sequence as shown in SEQ ID NO: 1.

[0013] In some aspects, the present invention provides a cultured cell comprising an expression vector comprising a polynucleotide of the present invention. In one embodiment, the polynucleotide comprises a nucleotide sequence as shown in SEQ ID NO: 1. In another embodiment, the cultured cell is a prokaryotic organism. In one embodiment, the prokaryotic organism is *E. coli*.

[0014] In one aspect, the present invention provides a method for making one or more recombinant plasmin polypeptides **having different N-termini.** The method comprises:

(a) providing **the above** polypeptide

(b) contacting the polypeptide provided in step a) with a protease under conditions sufficient to cleave one or more peptide bonds thereby forming the one more recombinant plasmin polypeptides **having different N-termini.** In one embodiment, providing comprises expressing an open reading frame having a sequence corresponding to the sequence as shown in SEQ ID NO: 1, or a degenerate variant thereof, in a suitable host. In another embodiment, the polypeptide has an amino acid sequence as shown in SEQ ID NO: 2.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015]

Figure 1 is a schematic representation of native plasmin after activation by proteolytic cleavage. K1-K5 are kringle regions 1-5; and SP is the serine protease domain. " α 2-AP" is the α 2-antiplasmin binding site on kringle 1.

Figure 2 is a schematic representation of a plasminogen deletion mutant of the invention using the same nomenclature as in Figure 1, and showing the deletion of K2-5.

Figure 3 shows the amino acid sequence of human plasminogen, showing the 19-residue leader sequence numbered as -19 to -1, and the plasminogen sequence shown as residues 1-791 (see SEQ ID NO: 3, the cDNA sequence for human plasminogen; and SEQ ID NO: 4, the encoded amino acid sequence, as shown in Figure 3). A number of features are shown, including the following: one embodiment of the (TAL6003)-plasminogen sequence (shaded); kringle domains 1-5 (double underscore); glycosylation sites Asn289 and Thr346 (in bold); the Arg-Val activation site (R561-V562 in bold); and lysine-binding sites in kringle 1 (in underscore and with specific position numbering). Figure 4 shows polypeptide sequence comparisons (i.e., a gap alignment) between the five kringle domains (1-5) of native human plasmin(ogen). Amino acid residues that are identical to those of the same relative position in kringle 1 are shown in underscore.

Figure 5 shows a 8-25% gradient SDS-PAGE of plasma-derived plasmin (Lane 1 = non-reduced (NR); Lane 2 = reduced (R)) and (TAL6003)-plasmin (Lane 3 = non-reduced (NR); Lane 4 = reduced (R)) preparation. Streptokinase activation of plasma-derived plasminogen and (TAL6003)-plasminogen into native plasmin and recombinant (TAL6003)-plasmin, respectively, results in the formation of two bands corresponding to the kringle and the serine protease domains. Accordingly, following incubation with the reducing agent dithiothreitol (DTT) prior to electrophoresis, plasma-derived plasmin and (TAL6003)-plasmin, which are a single band on a non-reduced gel, reduce to two bands corresponding to kringle 1 (lower band) and the serine protease domain (upper band) in the same non-reduced gel.

Figure 6 is a graphic representation of activation of (TAL6003)-plasminogen by streptokinase.

Figure 7 is a chromatogram showing binding of (TAL6003)-plasminogen to lysine-SEPHAROSE™ 4B: 0.5 mg of purified (TAL6003)-plasminogen was applied on the lysine-SEPHAROSE™ 4B column (1 × 3 cm) equilibrated with Tris-buffered saline, pH 7.4. Bound protein was eluted from the column by a 0-20 mM gradient of ϵ -aminocaproic acid (ϵ -ACA) as a single peak. The absorbance at 280 nm and the concentration of ϵ -ACA, as a function of the effluent volume are presented on the graph.

Figure 8 shows binding of (TAL6003)-plasminogen to fibrin as assessed by its subsequent activation by tPA and resulting clot lysis.

Figure 9 shows in vitro comparison of the thrombolytic efficacy of (TAL6003)-plasmin with plasma-derived plasmin. Figure 10 illustrates disulfide bonding pattern of (TAL6003)-plasmin (SEQ ID NO: 2). In the figure, (X) represents the amino acid sequence RDVVLFEK.

DESCRIPTION OF THE INVENTION

[0016] The present inventors have discovered novel recombinant plasminogen polypeptides, or variants thereof, herein referred to as (TAL6003)-plasminogens that have native plasminogen-like features despite deletion of 4 kringles from its structure. These (TAL6003)-plasminogens, or variants thereof, are zymogens that are capable of becoming activated to functional plasmin enzymes (herein referred to as (TAL6003)-plasmins) following an activation event that at least involves proteolytic cleavage of an Arg-Val peptide bond located between the kringle domain and the serine protease domain of the zymogen.

[0017] The (TAL6003)-plasminogen, or a variant thereof, of the present invention has fibrin- and antiplasmin-binding as well as activation properties of full-length native human plasminogen. Further, the (TAL6003)-plasminogen has a number of novel and desirable features including high-level expression in recombinant production and certain protein chemical structures identical or very similar to naturally occurring forms of human plasma-derived plasminogen.

[0018] The (TAL6003)-plasmin(ogen)s according to the present invention can be characterized at least by the following:

- (i) the lower molecular weights (e.g., in one embodiment about 36,911 to about 37,039 Da) of (TAL6003)-plasmins created following activation of (TAL6003)-plasminogens result in increased specific activity (per mg of protein);
- (ii) the lack of at least two glycosylation sites found in the native protein (*see* Figure 3, i.e., N289 and T346), combined with the relatively low molecular weights, facilitates recombinant production of this protein using relatively inexpensive bacterial and yeast expression systems;
- (iii) (TAL6003)-plasminogens can be activated by plasminogen activators tPA, urokinase, and streptokinase;
- (iv) the presence of the single N-terminal kringle domain homologous to a kringle domain of native human plasminogen preserves the fibrin-binding properties of plasmin which are important for thrombolytic efficacy;
- (v) the presence of α 2-antiplasmin-binding sites on the single N-terminal kringle domain homologous to a kringle domain of native human plasminogen allows the (TAL6003)-plasmins to be inhibited rapidly by this physiological inhibitor of plasmin (a feature which can prevent bleeding);
- (vi) the smaller size of the (TAL6003)-plasmins can facilitate their inhibition by α 2-macroglobulin, further lessening the chance of bleeding complications relative to native plasmin. In particular embodiments, the absence of kringle 5, which retains the primary binding site for intact, undigested fibrin(ogen), can allow use of the (TAL6003)-plasmins with reduced depletion of circulating fibrinogen;
- (vii) the presence of a single N-terminal kringle domain homologous to a kringle domain of native human plasminogen, wherein the last four amino acid residues within the kringle domain are V, P, Q, and C, provides a native-like linkage to the serine protease domain (i.e., a linkage similar to the naturally occurring domain juncture between the kringle 5 domain and the serine protease domain of human plasminogen); and
- (viii) following expression of the recombinant (TAL6003)-plasminogen, its N-terminus may be cleaved back (e.g., cleaved back during activation) to provide a native-like N-terminus.

[0019] Generally, the invention provides recombinant (TAL6003)-plasmin(ogen) polypeptides having a single kringle region N-terminal to the activation site and serine protease domain, having certain advantages relative to mini-plasmin(ogen). Although the (TAL6003)-plasminogens of the invention only have one kringle domain, as such, N-terminal to the activation site, some embodiments include additional sequences N-terminal to the activation site. Additional N-terminal sequences can be derived from those of native kringle regions of plasminogen.

[0020] The N-terminal kringle domains of the present invention include kringle sequences of kringles 1 and 4 of native plasmin(ogen) and functional equivalents thereof. In particular, *see* the discussion below that provides guidance regarding preservation of function in polypeptide variants, including preservation of residues participating in or influencing lysine binding.

[0021] Further, particular embodiments of the polypeptides of the present invention can exhibit reduced immunogenicity by virtue of native-like structures. For example, in some embodiments, the recombinant plasminogen of the present invention has an N-terminus identical to that of one of the naturally occurring forms of human plasma-derived plasminogen, which upon activation by streptokinase, produces plasmin polypeptides comprising native-like N-termini. Additionally, the novel polypeptides of the present invention have a sequence between the Kringle and Serine protease domains that is similar to the junction between Kringle 5 and the SP domain in naturally occurring human plasmin.

Definitions

[0022] The terms "domain" and "region" of a polypeptide are generally synonymous as used herein, unless otherwise indicated to the contrary. When recited together with well-recognized structural or functional designations such as "kringle" or "serine protease," etc., such terms will introduce a polypeptide feature relating to at least some characteristic(s) commonly recognized and understood to be associated with the polypeptide structures corresponding to such designa-

tions.

[0023] A "cultured host cell," as used herein, refers to a prokaryotic or eukaryotic cell that contains heterologous DNA that has been introduced into the cell by any means, e.g., electroporation, calcium phosphate precipitation, microinjection, transformation, viral infection, and the like.

[0024] "Heterologous" as used herein means "of different natural origin" or representing a non-natural state. For example, if a cultured host cell is transformed with a DNA or gene derived from another organism, particularly from another species, that gene is heterologous with respect to that cultured host cell, and also with respect to descendants of the cultured host cell which carry that gene. Similarly, "heterologous" refers to a nucleotide sequence derived from and inserted into the same natural, original cell type, but which is present in a non-natural state, e.g., a different copy number or under the control of different regulatory elements. Further, when used in the context of a nucleic acid or amino acid sequence, the term "heterologous" also may refer to any region of the sequence that is of a different natural origin than another region of the same sequence. For example, if a recombinant protein comprises a kringle domain derived from apolipoprotein(a) and a serine-protease domain derived from plasminogen, the kringle domain and the serine protease domain are "heterologous" relative to each other, particularly if each domain is derived from a different species or organism.

[0025] A "vector" molecule is a nucleic acid molecule into which heterologous nucleic acid can be inserted which can then be introduced into an appropriate cultured host cell. Vectors preferably have one or more origins of replication, and one or more sites into which the recombinant DNA can be inserted. Vectors often have convenient means by which cells with vectors can be selected from those without, e.g., they encode drug resistance genes. Common vectors include plasmids, viral genomes, and (primarily in yeast and bacteria) "artificial chromosomes."

[0026] As used herein, the term "transcriptional control sequence" refers to nucleic acid sequences, such as initiator sequences, enhancer sequences and promoter sequences, which induce, repress, or otherwise control the transcription of protein encoding nucleic acid sequences to which they are operably-linked.

[0027] The term "polypeptide" is used interchangeably herein with the terms "peptide" and "protein."

[0028] The terms "polynucleotide" and "nucleic acid" are used interchangeably herein, and can refer to any nucleic acid that contains the information necessary for the purpose indicated by the context. That is, the nucleic acid can be DNA or RNA, either single stranded or double stranded, or other nucleic acid, as long as the polymer is capable of representing the appropriate information, e.g., in relation to an encoded peptide, and can include complementary sequences, e.g., sense strands and anti-sense strands of nucleic acids polymers.

[0029] The term "variant" of a polypeptide refers to an amino acid sequence that is altered by one or more amino acids. The variant can have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties, e.g., replacement of leucine with isoleucine. Alternatively, a variant can have "non-conservative" changes, e.g., replacement of a glycine with a tryptophan. Analogous minor variation can also include amino acid deletion or insertion, or both. A particular form of a "variant" polypeptide is a "functionally equivalent" polypeptide, i.e., a polypeptide that exhibits substantially similar in vivo or in vitro activity as the examples of the polypeptide of invention, as described in more detail below. Guidance in determining which amino acid residues can be substituted, inserted, or deleted without eliminating biological or immunological activity can be found using computer programs well known in the art, for example, DNASTAR software (DNASTAR, Inc., Madison, WI). Further, specific guidance is provided below, including that provided within the cited references that are fully incorporated herein by reference.

[0030] The terms "N-terminal" and "C-terminal" are used herein to designate the relative position of any amino acid sequence or polypeptide domain or structure to which they are applied. The relative positioning will be apparent from the context. That is, an "N-terminal" feature will be located at least closer to the N-terminus of the polypeptide molecule than another feature discussed in the same context (the other feature possible referred to as "C-terminal" to the first feature). Similarly, the terms "5'" and "3'" can be used herein to designate relative positions of features of polynucleotides.

[0031] The polypeptides referred to herein as having an N-terminal domain "homologous to a kringle domain of native human plasminogen" exhibit structural and functional characteristics similar to native kringle domains of plasminogen. Further, the polypeptides referred to herein as having an N-terminal domain "homologous to kringle 1" exhibit characteristics similar to native kringle 1, at least to the extent that the polypeptides can have a higher affinity for ω -aminocarboxylic acids (and functional homologs such as *trans*-4 aminomethylcyclohexane-1-carboxylic acid, a cyclic acid) than kringle 5. See, e.g., Chang, Y., et al., *Biochemistry* 37: 3258-3271 (1998), incorporated herein by reference, for conditions and protocols for comparison of binding of isolated kringle domain polypeptides to 5-aminopentanoic acid (5-APnA); 6-aminohexanoic acid (6-AHxA), also known as ϵ -aminocaproic acid (ϵ -ACA); 7-aminoheptanoic acid (7-AHpA); and *trans*-4-aminomethylcyclohexane-1-carboxylic acid (t-AMCHA).

[0032] References to kringle domains "homologous to kringle 4" are defined similarly, as noted above regarding the phrase "homologous to kringle 1." That is, they exhibit functional characteristics similar to kringle 4 of native human plasminogen as discussed above. These polypeptides also bind immobilized lysine as described above.

[0033] The polypeptides of the invention bind immobilized lysine. As used herein, the phrase "binding immobilized lysine" means that the polypeptides so characterized are retarded in their progress relative to mini-plasminogen when

subjected to column chromatography using lysine-SEPHAROSE™ as the chromatographic media. Typically, the polypeptides of the invention can be eluted from such chromatographic media (lysine affinity resins) using solutions containing the specific ligand, e.g., ϵ -ACA, as eluants.

[0034] Further, in addition to Chang et al., *supra*, other references can be consulted by those of skill in the art to determine which residues can be varied by conservative or non-conservative substitution, deletion, or addition to yield a deletion mutant within the scope of the present invention. For example, the following references provide information regarding particular residues of the native kringle domains that may be important for binding of ω -aminocarboxylic acids: U.S. Pat. No. 6,538,103 to Ji, et al.; U.S. Pat. No. 6,218,517 to Suzuki; Douglas, J.T., et al., *Biochemistry* 41(10): 3302-10 (2002); Zajicek, J., et al., *J. Mol. Biol.* 301(2): 333-47 (2000); Lee, H., et al., *Arch Biochem Biophys.* 375(2): 359-63 (2000); Castellino, F. and S. McCance, *Ciba Found Symp.* 212: 46-60 (1997); McCance, S., et al., *J. Biol. Chem.* 269: 32405-32410 (1994); Rejante, M.R. and M. Llinas, *Eur. J. Biochem.* 221(3): 939-49 (1994); Wu, T.P., et al., *Blood Coagul. Fibrinolysis* 5(2): 157-66 (1994); Hoover, C.J., et al., *Biochemistry* 32(41): 10936-43 (1993); Menhart, N., et al., *Biochemistry* 32: 8799-8806 (1993); Thewes, T., et al., *J. Biol. Chem.*, 265 (7): 3906-3915 (1990); Novokhatny, V., et al., *Thromb Res.* 53(3): 243-52 (1989); Motta, A., et al., *Biochemistry* 26(13): 3827-36 (1987); Novokhatny, V., et al., *J. Mol. Biol.* 179: 215-232 (1984); Lerch, P.G., et al., *Eur. J. Biochem.* 107(1): 7-13 (1980); Sottrup-Jensen, L., et al., *Prog. Chem. Fibrinol. Thrombol.* 3: 191-209 (1978); and Wiman, B. and D. Collen, *Nature* 272: 549-545 (1978), all incorporated herein by reference in their entirety.

[0035] Because the present inventors have recognized that a valuable, simplified plasmin(ogen) molecule can be prepared having a single N-terminal kringle domain having advantageous functional characteristics (which can be evaluated, in part, by testing for the binding of immobilized lysine as described herein), the present invention can encompass other fibrin-binding domains or regions N-terminal to the activation site. For example, the invention can include polypeptides in which the serine protease domain of plasmin is attached to a fibrin-binding kringle selected from a group including, but not limited to, kringle 4 of human plasminogen, kringle 2 of tPA, or a kringle of apolipoprotein(a). Further, the invention can include polypeptides in which a serine protease domain of plasmin is attached to any other known fibrin-binding modules, such as the "finger" domain of tPA or fibronectin, or the FAB fragment of fibrin-specific IgG.

[0036] In some aspects, the polypeptides of the present invention have protein chemical structures (e.g., native-like N-terminus and native-like juncture between the kringle and the serine protease domain) that are identical to the chemical structures found in the naturally occurring forms of human plasma-derived plasmin(ogen). Without being held to a particular theory, it is believed that certain features of a protein can contribute to its immunogenicity, including but not limited to its amino acid sequence. Accordingly, the present invention provides an effective protein therapeutic based on recombinant (TAL6003)-plasminogen by preemptively reducing the potential immunogenicity of (TAL6003)-plasminogen through incorporation of amino acid sequences that resemble native human plasminogen sequences.

[0037] In one aspect, the recombinant (TAL6003)-plasminogen polypeptide of the present invention comprises (a) a single N-terminal kringle domain homologous to a kringle domain of native human plasminogen, wherein the last four amino acid residues within the kringle domain are V, P, Q, and C; and (b) a C-terminal domain activation site and serine protease domain homologous to the corresponding domains in human plasminogen; wherein the polypeptide binds to immobilized lysine **and is capable of becoming activated to functional plasmin enzymes following an activation event that at least involves proteolytic cleavage of an Arg-Val peptide bond located between the kringle domain and the serine protease domain.**

[0038] The recombinant (TAL6003)-plasminogen polypeptides of the present invention can be activated by one of ordinary skill in the art to provide a (TAL6003)-plasmin polypeptide. In one embodiment, the (TAL6003)-plasmin polypeptide exhibits a fibrinolytic activity that is inhibited by α 2-antiplasmin at a rate of inhibition that is at least about 5-fold faster than the rate of inhibition of the fibrinolytic activity of mini-plasmin by α 2-antiplasmin.

[0039] In some embodiments, the polypeptide has an amino acid sequence as shown in SEQ ID NO: 2, and conservative substitutions thereof. In other embodiments, the polypeptide has an arginine residue at a relative position analogous to that of position 85 of the amino acid sequence shown in SEQ ID NO: 2.

[0040] For example, the (TAL6003)-plasminogen described in this invention makes use of amino acid residue modifications to the junction region joining the single kringle domain and the serine protease domain. Accordingly, this juncture between the two domains more closely resembles the naturally occurring juncture between the kringle 5 domain and the serine protease domain of human plasminogen.

[0041] In another embodiment, the (TAL6003)-plasminogen described in this invention further comprises a native-like N-terminal sequence. The recombinantly produced (TAL6003)-plasminogen can be cleaved off upon activation to provide recombinant (TAL6003)-plasmin polypeptides also having native-like N-termini.

[0042] In particular embodiments, residues at certain positions of the single N-terminal kringle domain of (TAL6003)-plasminogen are conserved relative to kringle 1 of native human plasminogen. These can be residues at positions associated with disulfide bridging and lysine binding, and include Cys84, Cys105, Cys133, Cys145, Cys157, and Cys162, and Pro136-Pro140, Pro143-Tyr146, and Arg153-Tyr156, respectively (positions numbered as shown in Figure 3). Additionally, particular embodiments of the invention can be characterized chemically by contrast to mini-

plasmin(ogen) which has an analogous domain composition (i.e., kringle-serine protease (K-SP) (see Sottrup-Jensen, L., et al., Progress in Chemical Fibrinolysis and Thrombolysis, Vol. 3, (Eds: J. F. Davidson, et al.) Raven Press, New York (1978)) but, *inter alia*, lacks an arginine (Arg) at a relative position analogous to that of position 85 of the amino acid sequence shown in SEQ ID NO: 2. In some embodiments, the (TAL6003)-plasminogen of the invention comprises

5 a single N-terminal kringle domain comprising an Arg residue at a relative position analogous to that of position 85 of the amino acid sequence shown in SEQ ID NO: 2. Non-limiting examples of a relative position analogous to that of position 85 of the amino acid sequence shown in SEQ ID NO: 2 include Arg(153), Arg(234), Arg(324), and Arg(426) positions of the amino acid sequence shown in SEQ ID NO: 4.

[0043] Characterization of the single N-terminal kringle domain of (TAL6003)-plasminogen as "N-terminal" means only that the domain is present N-terminal to the activation site and does not mean that additional amino acids residues N-terminal to the domain itself are not present. Further, the number and identity of residues interposed between the most C-terminal cysteine residue of the single N-terminal kringle domain (i.e., the most C-terminal Cys residue shown in Figure 4) and the activation site of plasminogen can be varied without departing from the scope of the present invention. One of skill in the art will be able to determine these variations that achieve the benefits of the invention (kringle 1-like binding

10 of α aminocarboxylic acids, without substantial increase in size of the deletion mutant or introduction of potentially problematic glycosylation sites) without undue experimentation based on the disclosure herein and the references cited herein for guidance regarding kringle 1 function and structure.

[0044] Accordingly, the invention relates to polynucleotides, polypeptides, recombinant methods for producing the polypeptides, vectors containing the polynucleotides, expression systems for producing the polypeptides, and cultured

20 host cells comprising such expression systems.

[0045] As noted, in one aspect, the invention relates to a polynucleotide encoding the polypeptide disclosed herein or a polypeptide having conservative amino acid substitutions thereof. Guidance regarding selection of "conservative" amino acid substitutions is provided in more detail below. In one embodiment, the polynucleotide is DNA.

[0046] In another aspect, the invention relates to cells containing at least one polynucleotide of the invention.

25 [0047] In one embodiment, the polynucleotide comprises the nucleotide sequence as shown in SEQ ID NO: 1. In other embodiments, the polypeptide comprises the amino acid sequence as shown in SEQ ID NO: 2.

Polynucleotides

30 [0048] The polynucleotides of the invention include variants that have substitutions, deletions, and/or additions that can involve one or more nucleotides. The variants can be altered in coding regions, non-coding regions, or both. Alterations in the coding regions can produce conservative or non-conservative amino acid substitutions, deletions, or additions. Especially preferred among these are silent substitutions, additions and deletions, which do not alter the properties and activities of the (TAL6003)-plasmin(ogen) protein or portions thereof. Also especially preferred in this regard are conservative substitutions (*see below*).

35 [0049] In one embodiment, the nucleic acid molecule of the present invention comprises a polynucleotide having a nucleotide sequence that encodes a polypeptide having the amino acid sequence shown in SEQ ID NO: 2. In other embodiments, the nucleic acid molecule comprises a polynucleotide having the nucleotide sequence shown in SEQ ID NO: 1, or a degenerate variant thereof.

40 [0050] Of course, traditional techniques of molecular biology, microbiology, and recombinant nucleic acid can also be used to produce the polynucleotides of the invention. These techniques are well known and are explained in, for example, Current Protocols in Molecular Biology, F. M. Ausubel, ed., Vols. I, II, and III (1997); Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989); DNA Cloning: A Practical Approach, D. N. Glover, ed., Vols. I and II (1985); Oligonucleotide Synthesis, M. L. Gait, ed. (1984); Nucleic Acid Hybridization, Hames and Higgins, eds. (1985); Transcription and Translation, Hames and Higgins, eds. (1984); Animal Cell Culture, R. I. Freshney, ed. (1986); Immobilized Cells and Enzymes, IRL Press (1986); Perbal, "A Practical Guide to Molecular Cloning"; the series, Methods in Enzymology, Academic Press, Inc. (1984); Gene Transfer Vectors for Mammalian Cells, J. H. Miller and M. P. Calos, eds., Cold Spring Harbor Laboratory (1987); and Methods in Enzymology, Wu and Grossman and Wu, eds., respectively, Vols. 154 and 155, all incorporated herein by reference.

50

Vectors and Cultured Host Cells

[0051] The present invention also relates to vectors which include the isolated nucleic acid molecules of the present invention, cultured host cells which are genetically engineered with the recombinant vectors, and the production of the

55 (TAL6003)-plasmin(ogen) polypeptides by recombinant techniques.

[0052] Recombinant constructs can be introduced into cultured host cells using well-known techniques such as infection, transduction, transfection, transvection, electroporation, and transformation. The vector can be, for example, a phage, plasmid, viral or retroviral vector. Retroviral vectors can be replication competent or replication defective. In the

latter case, viral propagation generally will occur only in complementing cultured host cells.

[0053] The polynucleotides can be joined to a vector containing a selectable marker for propagation in a cultured host. Generally, a plasmid vector is introduced in a precipitate, such as a calcium phosphate precipitate, or in a complex with a charged lipid. If the vector is a virus, it can be packaged in vitro using an appropriate packaging cell line and then transduced into cultured host cells.

[0054] Preferred are vectors comprising *cis*-acting control regions to the polynucleotide of interest. Appropriate trans-acting factors can be supplied by the cultured host, supplied by a complementing vector, or supplied by the vector itself upon introduction into the cultured host.

[0055] In certain embodiments in this regard, the vectors provide for specific expression, which can be inducible and/or cell type-specific. Particularly preferred among such vectors are those inducible by environmental factors that are easy to manipulate, such as temperature and nutrient additives.

[0056] Expression vectors useful in the present invention include chromosomal-, episomal- and virus-derived vectors, e.g., vectors derived from bacterial plasmids, bacteriophage, yeast episomes, yeast chromosomal elements, viruses such as baculoviruses, papova viruses, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as cosmids and phagemids.

[0057] DNA inserts should be operatively linked to an appropriate promoter, such as the phage lambda PL promoter, the *E. coli* lac, trp and tac promoters, the SV40 early and late promoters and promoters of retroviral LTRs, to name a few. Other suitable promoters will be known to the skilled artisan. The expression constructs will further contain sites for transcription initiation, termination and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs can include a translation initiating at the beginning and a termination codon (UAA, UGA, or UAG) appropriately positioned at the end of the polypeptide to be translated.

[0058] As indicated, the expression vectors will preferably include at least one selectable marker. Such markers include dihydrofolate reductase or neomycin resistance for eukaryotic cell culture and tetracycline or ampicillin resistance genes for culturing in *E. coli* and other bacteria. Representative examples of appropriate cultured hosts include, but are not limited to, bacterial cells, such as *E. coli*, *Streptomyces* and *Salmonella typhimurium* cells; fungal cells, such as yeast cells; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS and Bowes melanoma cells; and plant cells. Appropriate culture mediums and conditions for the above-described cultured host cells are known in the art.

[0059] Among vectors preferred for use in bacteria include e.g., pET24b or pET22b available from Novagen, Madison, WI (pET-24b(+) and pET-22b(+)) = pET Expression System 24b (Cat. No. 69750) and 22b (Cat. No. 70765), respectively, EMD Biosciences, Inc., Novagen Brand, Madison, WI; see product information section regarding pET-24b and pET-22b for details regarding vector), pQE70, pQE60 and pQE-9, available from Qiagen Inc., Valencia, CA; pBS vectors, PHA-GESCRIP vectors, BLUESCRIPT vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene, LaJolla, CA; and ptc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia (now Pfizer, Inc., New York, NY). Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. Other suitable vectors will be readily apparent to the skilled artisan.

[0060] Bacterial promoters suitable for use in the present invention include the *E. coli* *lacI* and *lacZ* promoters, the T3 and T7 promoters, the gpt promoter, the lambda PR and PL promoters, and the trp promoter. Suitable eukaryotic promoters include the CMV immediate early promoter, the HSV thymidine kinase promoter, the early and late SV40 promoters, the promoters of retroviral LTRs, such as those of the Rous sarcoma virus (RSV), and metallothionein promoters, such as the mouse metallothionein-I promoter.

[0061] Introduction of a vector construct into the cultured host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection, or other methods. Such methods are described in many standard laboratory manuals, such as Davis et al., Basic Methods In Molecular Biology, 2nd Edition (1995).

[0062] Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes can be increased by inserting an enhancer sequence into the vector. Enhancers are *cis*-acting elements of DNA, usually about from 10 to 300 bp that act to increase transcriptional activity of a promoter in a given cultured host cell-type. Examples of enhancers include the SV40 enhancer, which is located on the late side of the replication origin at bp 100 to 270, the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

[0063] For secretion of the translated protein into the lumen of the endoplasmic reticulum, into the periplasmic space or into the extracellular environment, appropriate secretion signals can be incorporated into the expressed polypeptide. The signals can be endogenous to the polypeptide or they can be heterologous signals.

[0064] The polypeptide can be expressed in a modified form, such as a fusion protein, and can include not only secretion signals, but also additional heterologous functional regions. For instance, a region of additional amino acids, particularly charged amino acids, can be added to the N-terminus, for example, the polypeptide to improve stability and

persistence in the cultured host cell, during purification, or during subsequent handling and storage. Also, peptide moieties can be added to the polypeptide to facilitate purification. Such regions can be removed prior to final preparation of the polypeptide. The additions of peptide moieties to polypeptides to engender secretion or excretion, to improve stability and to facilitate purification, among others, are familiar and routine techniques in the art. A preferred fusion protein comprises a heterologous region from immunoglobulin that is useful to solubilize proteins. For example, EP 0 464 533 A1 (Canadian counterpart, 2,045,869) discloses fusion proteins comprising various portions of constant region of immunoglobulin molecules together with another human protein or part thereof. In many cases, the Fc part in a fusion protein is thoroughly advantageous for use in therapy and diagnosis and thus results, for example, in improved pharmacokinetic properties. On the other hand, for some uses it would be desirable to be able to delete the Fc part after the fusion protein has been expressed, detected, and purified in the advantageous manner described. This is the case when Fc portion proves to be a hindrance to use in therapy and diagnosis, for example, when the fusion protein is to be used as antigen for immunizations. In drug discovery for example, human proteins have been fused with Fc portions for the purpose of high-throughput screening assays (such as hIL5-receptor, to identify antagonists of hIL-5). See, Bennett, D., et al., J. Molecular Recognition, 8: 52-58 (1995) and Johanson, K. et al., J. Biol. Chem. 270(16): 9459-9471 (1995).

[0065] (TAL6003)-plasminogen can be recovered and purified from recombinant cell cultures by well-known methods including those specifically described in the examples herein. Polypeptides of the present invention include naturally purified products, products of chemical synthetic procedures, and products produced by recombinant techniques from a prokaryotic or eukaryotic cultured host, including, for example, bacterial, yeast, higher plant, insect and mammalian cells. In addition, polypeptides of the invention can also include an initial modified methionine residue, in some cases as a result of host-mediated processes.

[0066] It is also contemplated that polypeptides useful in production of the "isolated polypeptides" of the invention can be produced by solid phase synthetic methods. See Houghten, R. A., Proc. Natl. Acad. Sci. USA 82: 5131-5135 (1985); and U.S. Pat. No. 4,631,211 to Houghten et al. (1986).

[0067] The polypeptides of the present invention can be provided in an isolated form. By "isolated polypeptide" is intended a polypeptide removed from its native environment. Thus, a polypeptide produced and/or contained within a recombinant cultured host cell is considered isolated for purposes of the present invention. Also intended, as "isolated polypeptides" are polypeptides that have been purified, partially or substantially, from a recombinant cultured host.

EXAMPLES

Expression Vector Design

[0068] The amino acid sequence for (TAL6003)-plasminogen is shown in SEQ ID NO: 2. A polynucleotide having the nucleotide sequence encoding (TAL6003)-plasminogen was codon-optimized for *E. coli* expression and mRNA stability to provide the DNA sequence as shown in SEQ ID NO: 1. This polynucleotide was cloned into the *Nde*I and *Bam*H1 sites of *E. coli* expression vector pET24b(+) (Novagen; Madison, WI) to produce cytosolic protein.

[0069] As illustrated in Table 1, expression in bacteria (e.g., *E. coli*) provided a recombinant (TAL6003)-plasminogen polypeptide having the amino acid sequence as shown in SEQ ID NO: 2 (i.e., a recombinant (TAL6003)-plasminogen with an N-terminal methionine (i.e., M1) immediately preceding the arginine amino acid residue (i.e., R2) corresponding to the arginine at position 70 (i.e., R70) of the native human plasminogen amino acid sequence shown in SEQ ID NO: 4 (see also, e.g., Fig. 3). Such a recombinant product was susceptible to further cleavage to yield additional proteins having different N-termini including a protein with an N-terminal lysine (i.e., K10) or valine (i.e., V11) corresponding, respectively, to the lysine at position 78 (i.e., K78) or the valine at position 79 (i.e., V79) of native human plasminogen.

Table 1: N-termini of native plasmin(ogen) (e.g., based on SEQ ID NO: 4) and (TAL6003)-plasmin(ogen) (e.g., based on SEQ ID NO: 2, or a variant thereof)

Native Plasminogen comprising 19 amino acid leader sequence (e.g., based on SEQ ID NO: 4):

M⁻¹⁹EHKE ... E⁰¹PLDDY ... M⁶⁹R⁷⁰DVVLFEKK⁷⁸V⁷⁹YLSEC

Native "Lys-Plasminogen" (i.e., cleavage of leader sequence):

E⁰¹PLDDY ... M⁶⁹R⁷⁰DVVLFEKK⁷⁸V⁷⁹YLSEC
(see SEQ ID NO:15)

Native Plasmin species possible based on cleavage, if any, of Lys-Plasminogen:

(see SEQ ID NO:14)

M⁶⁹R⁷⁰DVVLFEKK⁷⁸V⁷⁹YLSEC

(continued)

Table 1: N-termini of native plasmin(ogen) (e.g., based on SEQ ID NO: 4) and (TAL6003)-plasmin(ogen) (e.g., based on SEQ ID NO: 2, or a variant thereof)

(see SEQ ID NO:13)	K ⁷⁸ V ⁷⁹ YLSEC
(see SEQ ID NO:12)	V ⁷⁹ YLSEC
Recombinant (TAL6003)-plasminogen polypeptides of the present invention: (e.g., based on SEQ ID NO: 2) <div style="text-align: center;"> ↓ ↓ M⁰¹R⁰²DVVLFEKK¹⁰V¹¹YLSEC </div>	
Additional proteins based on further cleavage of a (TAL6003)-plasminogen (e.g., based on SEQ ID NO: 2): (see SEQ ID NO:11)K ¹⁰ V ¹¹ YLSEC (SEQ ID NO:5) V ¹¹ YLSEC	
↓ indicates potential cleavage sites.	

(TAL6003)-plasminogen Expression and Purification

[0070] The expression vector comprising the DNA encoding (TAL6003)-plasminogen was transformed into a variety of cells including BL21(DE3) RIL (Stratagene, La Jolla, CA), BL21(DE3) (genotype: F^{ompT} hsdSB (rB⁻ mB⁻) gal dcm (DE3)) (EMB Biosciences, Inc., San Diego, CA), and BLR(DE3) (genotype: F^{ompT} hsdSB (rB⁻ mB⁻) gal dcm (DE3) Δ (srI⁻ recA)306::Tn10(TetR)), and protein over-expression following induction by 1 mM IPTG (isopropyl-beta-D-thiogalactopyranoside) was analyzed by SDS-PAGE. Expression estimates were at least about 250 mg/L cell culture in shaker flasks.

[0071] Cell type BL21(DE3) RIL is engineered to express rare *E. coli* tRNAs coding for Arg, Ile, and Leu. Further, both BL21(DE3) and BLR(DE3) are B strain *E. coli* that is classified as non-pathogenic to humans and animals based on the absence of virulence and colonization factors. BLR(DE3) cells lack the recA gene for DNA recombination, and induction of lambda phage has not been reported with these cells. A research cell bank of the (TAL6003)-plasminogen construct in BLR(DE3) cells was produced and tested for purity, identity, and induction of bacteriophage at Charles River Laboratories (Malvern, PA). The testing confirmed the identity and purity of the research cell bank and the cells passed the phage induction test with no phage observed (data not shown).

[0072] Production of (TAL6003)-plasminogen (i.e., based on SEQ ID NO: 2) was confirmed in larger scale expression in which cells were lysed and both soluble protein and purified inclusion bodies were examined by SDS-PAGE.

[0073] The following typical protocol has been used for expression of (TAL6003)-plasminogen:

A single colony of *E. coli* cells (e.g., BL21(DE3) RIL, BL21(DE3), or BLR(DE3) containing the (TAL6003)-plasminogen vector was used to inoculate 5 mL of LB/kanamycin (30 μg/mL) and was incubated for 8 hours at 37 °C on a shaker. After that, a 50 μL-aliquot was taken from the cultured bacterial suspension for further growth in fresh media. The procedure was repeated after 16 hours with 6 mL of bacterial culture and 250 mL of the media. Cultures were grown at 37 °C with shaking to an OD_{600 nm} of ~1.0, and IPTG was added to 1 mM final concentration. Cultures were grown for an additional 5 hours. Cells were harvested by centrifugation at 5,000 × g and cell pellets were dissolved in 20 mM Tris pH 8.0 containing 20 mM EDTA and frozen at -80°C.

[0074] To purify (TAL6003)-plasminogen, cell pellets were thawed and buffer added until the solution volume was approximately 1/20th that of the original cell culture volume. After that, lysozyme was added to a final concentration of 0.5 mg/mL and the cells were stirred rapidly at 4 °C for 10-15 minute. Then, Triton X-100 was added to 1% final concentration and stirring continued for another 10 min. DNase I (0.05 mg/mL) and MgCl₂ (2.5 mM) were added and stirring was continued at 4 °C for 30 minutes or until the solution was no longer viscous. The final solution was centrifuged at 4 °C for 30 min at 15,000 × g and the supernatant was discarded.

[0075] The cell pellet was washed three times with wash solution (50 mM Tris-HCl, pH 7.4 containing 10 mM EDTA, 1% Triton-X-100, and 0.5 M urea), and the final pellet was dissolved in 40 mL of extraction buffer (PBS, pH 7.4 containing 10 mM EDTA, 20 mM DTT, and 6 M guanidine-HCl) and stored at 4 °C overnight. After 16 hours, the solution was centrifuged for 30 minutes at 15,000 × g to remove solids and the supernatant was slowly added to the refolding solution (50 mM Tris-HCl, pH 8.3, 3.5 M guanidine-HCl, 0.5 M arginine-HCl, 10 mM EDTA, 3 mM GSH, 0.3 mM GSSG) while stirring at 4 °C. The refolding procedure was carried out at protein concentration of about 0.29 g/L.

[0076] The refolding solution was kept for 2 days at 4 °C undisturbed and then dialyzed against an 8-fold volume of 0.1 M Tris-HCl pH 8.0 containing 10 mM EDTA, 0.15 M NaCl, 0.15 M arginine-HCl, over a period of 8-10 hours with

frequent changes of the buffer solution.

[0077] The protein solution was then removed from dialysis and concentrated using AMICON filters with the membrane cut-off of 10 kDa to approximately 10-20 mL and dialyzed overnight versus a 100-fold volume of 0.1 M Tris pH 8.0 containing 10 mM EDTA, 0.15 M NaCl. This material was centrifuged to remove particulates, and then passed over lysine affinity resin (Lysine-SEPHAROSE™ 4B; Amersham Biosciences, Piscataway, NJ). (TAL6003)-plasminogen was eluted from the resin using Tris-buffered saline, pH 8.0 containing 0.2 M epsilon aminocaproic acid (ϵ -ACA).

[0078] Typically, 80 mg of inclusion bodies could be isolated from 1 liter of cell culture and 40 mg could be eluted in the lysine-SEPHAROSE chromatography step.

Properties of (TAL6003)-plasminogen

[0079] Purified (TAL6003)-plasminogen appeared as a band in the 35-40 kDa region by SDS-PAGE analysis of reduced (dithiothreitol-treated) and non-reduced protein. Its molecular mass, determined by MALDI mass-spectrometry, was about 38,140 Da, which is close to the expected value.

[0080] To determine the rate of activation of (TAL6003)-plasminogen by streptokinase, 1 mg/mL of recombinant (TAL6003)-plasminogen was mixed with streptokinase at a 1:100 (TAL6003)-plasminogen to streptokinase ratio and incubated at room temperature at pH 7. At various time points, samples were removed and quenched with SDS-Page buffer and analyzed on reduced SDS-PAGE followed by densitometry to determine the conversion of the one-chain (TAL6003)-plasminogen molecule into a two-chain (TAL6003)-plasmin. Percent activation of (TAL6003)-plasminogen by streptokinase is shown in Figure 6 as loss of full-length (TAL6003)-plasminogen over time as determined by SDS-PAGE.

[0081] To confirm the functionality of kringle 1, we determined the binding of TAL6003-plasminogen to lysine-SEPHAROSE™ 4B. As shown in Figure 7, (TAL6003)-plasminogen bound to lysine-SEPHAROSE™ and could be eluted from the column by a 0-20 mM ϵ -ACA gradient as a single peak at about 4 mM. The ability of refolded (TAL6003)-plasminogen to bind lysine-SEPHAROSE™ indicates that the kringle domain of the molecule is properly folded and the lysine-binding site is fully active.

[0082] To further confirm the functionality of kringle 1, the binding of ϵ -ACA to (TAL6003)-plasminogen was measured by monitoring the associated changes in protein fluorescence as described by Matsuka et al., Eur. J. Biochem. 190: 93-97 (1990) and Douglas et al., J. Biochemistry 41: 3302-3310 (2002), all incorporated herein by reference. Binding of ϵ -ACA to kringle 1 of (TAL6003)-plasminogen results in a decrease in fluorescence, likely due to quenching of the tryptophan residues which are part of the lysine-binding site.

[0083] To monitor this process, 4 μ L to 16 μ L aliquots of a concentrated solution of ϵ -ACA were added to 2 mL of 5 μ M (TAL6003)-plasminogen in 50 mM Tris buffer containing 20 mM NaCl, pH 8.0, 25 °C. The fluorescence was monitored at an excitation wavelength of 298 nm and an emission wavelength of 340 nm in a FLUOROMAX fluorescence spectrophotometer (Jobin Yvon, Inc., Edison, NJ); after each addition of ϵ -ACA, the solution was allowed to equilibrate until no further changes in fluorescence were observed.

[0084] The resulting fluorescence values were corrected for dilution and plotted versus the concentration of ϵ -ACA over a range of 0-50 μ M ϵ -ACA. Data were fitted by non-linear regression to obtain a K_d of about 19 μ M.

[0085] One property of plasminogen is its ability to bind fibrin. In order to determine whether (TAL6003)-plasminogen retains the ability to interact with fibrin, its fibrin-binding properties was tested in a microtiter plate assay in which binding of (TAL6003)-plasminogen to fibrin was assessed by its subsequent activation by tPA and resulting clot lysis. For this purpose, 100 μ L of 5 mg/mL fibrinogen was polymerized with thrombin in each well of a microtiter plate. Various concentrations of (TAL6003)-plasminogen was added on top of the fibrin clots and incubated for 1 hour at 37 °C. The plate was washed extensively with PBS while the fibrin clots are still intact and attached to the wells. After washing, a 0.1 mg/mL solution of tPA was added to each well and the plate was incubated 2 hours at 37 °C. As a result, some of the clots were completely dissolved and some were partially dissolved, while wells with very low amounts of (TAL6003)-plasminogen and control wells remained practically intact. The degree of fibrinolysis was monitored by measuring the 280 nm absorbance of remainders of the initial clots reconstituted in 1 M NaOH. The absorbance values were plotted as a function of (TAL6003)-plasminogen concentration.

[0086] As shown in Figure 8, the binding of (TAL6003)-plasminogen to fibrin followed a classic, sigmoidal binding curve. Using this assay, it was found that (TAL6003)-plasminogen binds fibrin with affinity comparable to that of full-length plasminogen and the C_{50} of this interaction (~ 0.3 μ M) is comparable to the K_d of fibrin-binding of full-length plasminogen.

[0087] These experiments indicate that (TAL6003)-plasminogen can bind fibrin. Further, at least the interaction of (TAL6003)-plasminogen with lysine-Sepharose, its ability to bind ϵ -ACA with the expected K_d , its ability to bind fibrin, and its ability to be activated by a plasminogen activator all indicated that this molecule was produced in the *E. coli* system in a fully functional form.

(TAL6003)-plasmin Purification and Formulation

[0088] The addition of SK to the purified (TAL6003)-plasminogen solution effects the conversion of (TAL6003)-plasminogen to (TAL6003)-plasmin. The protein was concentrated to 2 mg/mL and diluted 1:1 with 50% glycerol to produce a 1 mg/mL solution in 25% glycerol. The solution was brought to room temperature and streptokinase was added at a 1:100 molar ratio of SK:(TAL6003)-plasminogen. The reaction was incubated without stirring at room temperature for 4.5 hrs. The reaction was then slowed down by addition of NaCl to a 0.5 M final concentration. Analysis of activation by SDS-PAGE indicated a 90% yield of activated protein.

[0089] Activated (TAL6003)-plasmin was purified by Benzamidine Affinity Chromatography. The purpose of benzamidine affinity purification was the separation of unactivated (TAL6003)-plasminogen and impurities, including (TAL6003)-plasmin degradation products, from active (TAL6003)-plasmin. The SK activation solution was applied to an equilibrated Benzamidine-SEPHAROSE™ 4 Fast Flow column. The (TAL6003)-plasmin, both clipped and intact, was captured by the affinity resin while the aforementioned impurities flowed through the column. The column was washed with the equilibration buffer until the absorbance at 280 nm reached baseline. The bound (TAL6003)-plasmin was then eluted using a low pH ϵ -ACA step to strip all remaining protein from the column. Typical yields were 75%, with protein that is 95% active as measured by chromogenic plasmin potency assay.

[0090] Because (TAL6003)-plasmin, similar to full-length plasmin, is prone to auto-degradation at physiological pH, pH 3.6 was chosen for the final formulation (acidified with acetic acid-saline). As shown previously for plasmin by Novokhatny et al., J. Thromb. Haemost. 1(5): 1034-41 (2003), incorporated by reference, and confirmed in experiments with (TAL6003)-plasmin, this low buffering-capacity, low pH formulation not only allows safe storage of active plasmins for prolonged periods of time, but is also compatible with parenteral administration of these direct thrombolytics. When mixed with plasma or neutral pH buffers, (TAL6003)-plasmin is quickly re-activated.

Enzymatic Properties of (TAL6003)-plasmin

[0091] The amidolytic activity of (TAL6003)-plasmin was examined using the plasmin substrate D-Val-Leu-Lys-p-nitroanilide (S-2251) (DiaPharma, West Chester, OH).

[0092] For (TAL6003)-plasmin, at pH 7.4, 25 °C in PBS buffer, the Michaelis-Menten constant (K_M) for S-2251 was found to also be 141 μ M (Table 2). The k_{cat} for the preparation was found to be about 725 min⁻¹. Using 4-nitrophenyl 4-guanidinobenzoate hydrochloride (pNPGb) titration (Chase, T. and E. Shaw, Methods Enzymol. 197: 20-27 (1970)), the percent of functional active sites was found to be 67%. Correcting k_{cat} for percent active sites, a k_{cat} of about 725 min⁻¹ was determined. This value was very close to the value determined in the same assay for full-length plasmin, 820 \pm 23 min⁻¹ and for micro-plasmin (lacking all five kringles), 795 \pm 24 min⁻¹. These data indicate that presence or absence of kringles does not affect the catalytic activity of the serine protease domain.

Table 2. Steady-state kinetic parameters for various plasmin species with substrate S-2251, in PBS buffer, pH 7.4, 25 °C.

	K_M	k_{cat}
plasmin	220 \pm 9 μ M	820 \pm 23 min ⁻¹
mini-plasmin	160 \pm 30 μ M	770 \pm 70 min ⁻¹
micro-plasmin	145 \pm 13 μ M	795 \pm 24 min ⁻¹
(TAL6003)-plasmin	141 \pm 9 μ M	725 min ⁻¹

[0093] The rate of inhibition of (TAL6003)-plasmin by α 2-antiplasmin was determined to be $1.8 \pm 0.06 \times 10^7$ M⁻¹ s⁻¹ using the method of Wiman and Collen (Wiman, B. and D. Collen, Eur. J. Biochem. 84: 573-578 (1978)) in which plasmin and α 2-antiplasmin are mixed then assayed for S-2251 activity at specific time points (Table 3). This value is comparable to reported values for plasmin of 2.5×10^7 M⁻¹ s⁻¹ (from Anonick, et al., Thrombosis Res. 59: 449 (1990)).

Table 3. Inhibition rates for various plasmin species and inhibitors were determined at 22 °C in PBS buffer, pH 7.4.

	α 2-antiplasmin
plasmin	$2.5 \pm 0.5 \times 10^7$ M ⁻¹ s ⁻¹
mini-plasmin	$2.4 \pm 0.5 \times 10^5$ M ⁻¹ s ⁻¹

(continued)

Table 3. Inhibition rates for various plasmin species and inhibitors were determined at 22 °C in PBS buffer, pH 7.4.

	α_2 -antiplasmin
micro-plasmin	$1.8 \pm 0.2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$
(TAL6003)-plasmin	$1.8 \pm 0.06 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$

[0094] The same experiments conducted with micro-plasmin revealed α_2 -antiplasmin inhibition rates of $1.8 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $3.1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ in two separate experiments. The rate of α_2 -antiplasmin inhibition of mini-plasmin (mini-plasmin domain composition, K5-SP) was determined to be $2.4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. These data are in reasonable agreement with literature values for micro- and mini-plasmin and show that inhibition of (TAL6003)-plasmin by α_2 -antiplasmin is 40-fold faster than the inhibition of either micro-plasmin or mini-plasmin. Thus, these results indicate that (TAL6003)-plasmin should be rapidly inhibited by α_2 -antiplasmin due to the presence of kringle 1 in its structure. Overall, the data presented in this section show that the enzymatic and inhibitory properties of (TAL6003)-plasmin is similar to full-length plasmin.

[0095] Literature values are taken from Anonick, et al., *Thrombosis Res.* 59: 449 (1990). All rates were measured according to the methods published in Anonick, et al.

In Vitro Fibrinolytic Efficacy

[0096] The fibrinolytic efficacy of (TAL6003)-plasmin was tested in an in vitro model of clot lysis assay using the following experimental protocol.

[0097] In vitro comparison of the thrombolytic efficacy of (TAL6003)-plasmin with plasma-derived plasmin. Equimolar amounts of plasma-derived plasmin (0.25 mg/mL) and (TAL6003)-plasmin (0.11 mg/mL) were mixed with blood clots in the test tube and degree of clot lysis was monitored by A_{280} absorbance of material released from the clot.

[0098] The concentrations of plasmin or (TAL6003)-plasmin required to overcome plasma inhibitors in the presence of fibrin and initiate clot lysis are shown in Figure 9.

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Novokhatny, Valery

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				100					105					110			
10	Pro	His	Arg	Pro	Arg	Phe	Ser	Pro	Ala	Thr	His	Pro	Ser	Glu	Gly	Leu	
			115					120					125				
15	Glu	Glu	Asn	Tyr	Cys	Arg	Asn	Pro	Asp	Asn	Asp	Pro	Gln	Gly	Pro	Trp	
		130					135					140					
20	Cys	Tyr	Thr	Thr	Asp	Pro	Glu	Lys	Arg	Tyr	Asp	Tyr	Cys	Asp	Ile	Leu	
	145					150					155					160	
25	Glu	Cys	Glu	Glu	Glu	Cys	Met	His	Cys	Ser	Gly	Glu	Asn	Tyr	Asp	Gly	
					165					170					175		
30	Lys	Ile	Ser	Lys	Thr	Met	Ser	Gly	Leu	Glu	Cys	Gln	Ala	Trp	Asp	Ser	
				180					185					190			
35	Gln	Ser	Pro	His	Ala	His	Gly	Tyr	Ile	Pro	Ser	Lys	Phe	Pro	Asn	Lys	
			195					200					205				
40	Asn	Leu	Lys	Lys	Asn	Tyr	Cys	Arg	Asn	Pro	Asp	Arg	Glu	Leu	Arg	Pro	
		210					215					220					
45	Trp	Cys	Phe	Thr	Thr	Asp	Pro	Asn	Lys	Arg	Trp	Glu	Leu	Cys	Asp	Ile	
	225					230					235					240	
50	Pro	Arg	Cys	Thr	Thr	Pro	Pro	Pro	Ser	Ser	Gly	Pro	Thr	Tyr	Gln	Cys	
					245					250					255		
55	Leu	Lys	Gly	Thr	Gly	Glu	Asn	Tyr	Arg	Gly	Asn	Val	Ala	Val	Thr	Val	
				260					265					270			
60	Ser	Gly	His	Thr	Cys	Gln	His	Trp	Ser	Ala	Gln	Thr	Pro	His	Thr	His	
			275					280					285				
65	Asn	Arg	Thr	Pro	Glu	Asn	Phe	Pro	Cys	Lys	Asn	Leu	Asp	Glu	Asn	Tyr	
		290					295					300					
70	Cys	Arg	Asn	Pro	Asp	Gly	Lys	Arg	Ala	Pro	Trp	Cys	His	Thr	Thr	Asn	
	305					310					315					320	

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	Ser	Gln	Val	Arg	Trp	Glu	Tyr	Cys	Lys	Ile	Pro	Ser	Cys	Asp	Ser	Ser	
					325					330					335		
5	Pro	Val	Ser	Thr	Glu	Gln	Leu	Ala	Pro	Thr	Ala	Pro	Pro	Glu	Leu	Thr	
				340					345					350			
10	Pro	Val	Val	Gln	Asp	Cys	Tyr	His	Gly	Asp	Gly	Gln	Ser	Tyr	Arg	Gly	
			355					360					365				
15	Thr	Ser	Ser	Thr	Thr	Thr	Thr	Gly	Lys	Lys	Cys	Gln	Ser	Trp	Ser	Ser	
		370					375					380					
20	Met	Thr	Pro	His	Arg	His	Gln	Lys	Thr	Pro	Glu	Asn	Tyr	Pro	Asn	Ala	
	385					390					395					400	
25	Gly	Leu	Thr	Met	Asn	Tyr	Cys	Arg	Asn	Pro	Asp	Ala	Asp	Lys	Gly	Pro	
					405					410					415		
30	Trp	Cys	Phe	Thr	Thr	Asp	Pro	Ser	Val	Arg	Trp	Glu	Tyr	Cys	Asn	Leu	
				420					425					430			
35	Lys	Lys	Cys	Ser	Gly	Thr	Glu	Ala	Ser	Val	Val	Ala	Pro	Pro	Pro	Val	
			435					440					445				
40	Val	Leu	Leu	Pro	Asp	Val	Glu	Thr	Pro	Ser	Glu	Glu	Asp	Cys	Met	Phe	
		450					455					460					
45	Gly	Asn	Gly	Lys	Gly	Tyr	Arg	Gly	Lys	Arg	Ala	Thr	Thr	Val	Thr	Gly	
	465					470					475					480	
50	Thr	Pro	Cys	Gln	Asp	Trp	Ala	Ala	Gln	Glu	Pro	His	Arg	His	Ser	Ile	
					485					490					495		
55	Phe	Thr	Pro	Glu	Thr	Asn	Pro	Arg	Ala	Gly	Leu	Glu	Lys	Asn	Tyr	Cys	
				500					505					510			
60	Arg	Asn	Pro	Asp	Gly	Asp	Val	Gly	Gly	Pro	Trp	Cys	Tyr	Thr	Thr	Asn	
			515					520					525				
65	Pro	Arg	Lys	Leu	Tyr	Asp	Tyr	Cys	Asp	Val	Pro	Gln	Cys	Ala	Ala	Pro	
		530					535					540					
70	Ser	Phe	Asp	Cys	Gly	Lys	Pro	Gln	Val	Glu	Pro	Lys	Lys	Cys	Pro	Gly	
	545					550					555					560	

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	Arg	Val	Val	Gly	Gly	Cys	Val	Ala	His	Pro	His	Ser	Trp	Pro	Trp	Gln	
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5	Val	Ser	Leu	Arg	Thr	Arg	Phe	Gly	Met	His	Phe	Cys	Gly	Gly	Thr	Leu	
				580					585					590			
10	Ile	Ser	Pro	Glu	Trp	Val	Leu	Thr	Ala	Ala	His	Cys	Leu	Glu	Lys	Ser	
			595					600					605				
15	Pro	Arg	Pro	Ser	Ser	Tyr	Lys	Val	Ile	Leu	Gly	Ala	His	Gln	Glu	Val	
		610					615					620					
20	Asn	Leu	Glu	Pro	His	Val	Gln	Glu	Ile	Glu	Val	Ser	Arg	Leu	Phe	Leu	
	625					630					635					640	
25	Glu	Pro	Thr	Arg	Lys	Asp	Ile	Ala	Leu	Leu	Lys	Leu	Ser	Ser	Pro	Ala	
					645					650					655		
30	Val	Ile	Thr	Asp	Lys	Val	Ile	Pro	Ala	Cys	Leu	Pro	Ser	Pro	Asn	Tyr	
				660					665					670			
35	Val	Val	Ala	Asp	Arg	Thr	Glu	Cys	Phe	Ile	Thr	Gly	Trp	Gly	Glu	Thr	
			675					680					685				
40	Gln	Gly	Thr	Phe	Gly	Ala	Gly	Leu	Leu	Lys	Glu	Ala	Gln	Leu	Pro	Val	
		690					695					700					
45	Ile	Glu	Asn	Lys	Val	Cys	Asn	Arg	Tyr	Glu	Phe	Leu	Asn	Gly	Arg	Val	
	705					710					715					720	
50	Gln	Ser	Thr	Glu	Leu	Cys	Ala	Gly	His	Leu	Ala	Gly	Gly	Thr	Asp	Ser	
					725					730					735		
55	Cys	Gln	Gly	Asp	Ser	Gly	Gly	Pro	Leu	Val	Cys	Phe	Glu	Lys	Asp	Lys	
				740					745					750			
60	Tyr	Ile	Leu	Gln	Gly	Val	Thr	Ser	Trp	Gly	Leu	Gly	Cys	Ala	Arg	Pro	
			755					760					765				
65	Asn	Lys	Pro	Gly	Val	Tyr	Val	Arg	Val	Ser	Arg	Phe	Val	Thr	Trp	Ile	
		770					775					780					
70	Glu	Gly	Val	Met	Arg	Asn	Asn										
	785					790											

<210> 6

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<211> 723
 <212> PRT
 <213> Homo sapiens

5 <400> 6

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10		Lys	Thr	Gly	Asn	Gly	Lys	Asn	Tyr	Arg	Gly	Thr	Met	Ser	Lys	Thr	Lys	
				20					25						30			
15	Asn	Gly	Ile	Thr	Cys	Gln	Lys	Trp	Ser	Ser	Thr	Ser	Pro	His	Arg	Pro		
			35					40					45					
	Arg	Phe	Ser	Pro	Ala	Thr	His	Pro	Ser	Glu	Gly	Leu	Glu	Glu	Asn	Tyr		
20		50					55					60						
	Cys	Arg	Asn	Pro	Asp	Asn	Asp	Pro	Gln	Gly	Pro	Trp	Cys	Tyr	Thr	Thr		
	65					70				75						80		
25		Asp	Pro	Glu	Lys	Arg	Tyr	Asp	Tyr	Cys	Asp	Ile	Leu	Glu	Cys	Glu	Glu	
					85					90						95		
30	Glu	Cys	Met	His	Cys	Ser	Gly	Glu	Asn	Tyr	Asp	Gly	Lys	Ile	Ser	Lys		
				100					105					110				
	Thr	Met	Ser	Gly	Leu	Glu	Cys	Gln	Ala	Trp	Asp	Ser	Gln	Ser	Pro	His		
35			115					120					125					
	Ala	His	Gly	Tyr	Ile	Pro	Ser	Lys	Phe	Pro	Asn	Lys	Asn	Leu	Lys	Lys		
		130					135					140						
40		Asn	Tyr	Cys	Arg	Asn	Pro	Asp	Arg	Glu	Leu	Arg	Pro	Trp	Cys	Phe	Thr	
	145					150						155				160		
45		Thr	Asp	Pro	Asn	Lys	Arg	Trp	Glu	Leu	Cys	Asp	Ile	Pro	Arg	Cys	Thr	
					165						170					175		
	Thr	Pro	Pro	Pro	Ser	Ser	Gly	Pro	Thr	Tyr	Gln	Cys	Leu	Lys	Gly	Thr		
50				180					185					190				
	Gly	Glu	Asn	Tyr	Arg	Gly	Asn	Val	Ala	Val	Thr	Val	Ser	Gly	His	Thr		
			195					200					205					
55		Cys	Gln	His	Trp	Ser	Ala	Gln	Thr	Pro	His	Thr	His	Asn	Arg	Thr	Pro	
		210						215					220					

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	Glu	Asn	Phe	Pro	Cys	Lys	Asn	Leu	Asp	Glu	Asn	Tyr	Cys	Arg	Asn	Pro	
	225					230					235					240	
5	Asp	Gly	Lys	Arg	Ala	Pro	Trp	Cys	His	Thr	Thr	Asn	Ser	Gln	Val	Arg	
					245					250					255		
10	Trp	Glu	Tyr	Cys	Lys	Ile	Pro	Ser	Cys	Asp	Ser	Ser	Pro	Val	Ser	Thr	
				260					265					270			
15	Glu	Gln	Leu	Ala	Pro	Thr	Ala	Pro	Pro	Glu	Leu	Thr	Pro	Val	Val	Gln	
			275					280					285				
20	Asp	Cys	Tyr	His	Gly	Asp	Gly	Gln	Ser	Tyr	Arg	Gly	Thr	Ser	Ser	Thr	
	290						295					300					
25	Thr	Thr	Thr	Gly	Lys	Lys	Cys	Gln	Ser	Trp	Ser	Ser	Met	Thr	Pro	His	
	305					310					315					320	
30	Arg	His	Gln	Lys	Thr	Pro	Glu	Asn	Tyr	Pro	Asn	Ala	Gly	Leu	Thr	Met	
					325					330					335		
35	Asn	Tyr	Cys	Arg	Asn	Pro	Asp	Ala	Asp	Lys	Gly	Pro	Trp	Cys	Phe	Thr	
				340					345					350			
40	Thr	Asp	Pro	Ser	Val	Arg	Trp	Glu	Tyr	Cys	Asn	Leu	Lys	Lys	Cys	Ser	
			355					360					365				
45	Gly	Thr	Glu	Ala	Ser	Val	Val	Ala	Pro	Pro	Pro	Val	Val	Leu	Leu	Pro	
		370					375					380					
50	Asp	Val	Glu	Thr	Pro	Ser	Glu	Glu	Asp	Cys	Met	Phe	Gly	Asn	Gly	Lys	
	385					390					395					400	
55	Gly	Tyr	Arg	Gly	Lys	Arg	Ala	Thr	Thr	Val	Thr	Gly	Thr	Pro	Cys	Gln	
					405					410					415		
60	Asp	Trp	Ala	Ala	Gln	Glu	Pro	His	Arg	His	Ser	Ile	Phe	Thr	Pro	Glu	
				420					425					430			
65	Thr	Asn	Pro	Arg	Ala	Gly	Leu	Glu	Lys	Asn	Tyr	Cys	Arg	Asn	Pro	Asp	
			435					440					445				
70	Gly	Asp	Val	Gly	Gly	Pro	Trp	Cys	Tyr	Thr	Thr	Asn	Pro	Arg	Lys	Leu	
	450						455					460					

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	Tyr	Asp	Tyr	Cys	Asp	Val	Pro	Gln	Cys	Ala	Ala	Pro	Ser	Phe	Asp	Cys	465	470	475	480
5	Gly	Lys	Pro	Gln	Val	Glu	Pro	Lys	Lys	Cys	Pro	Gly	Arg	Val	Val	Gly		485	490	495
10	Gly	Cys	Val	Ala	His	Pro	His	Ser	Trp	Pro	Trp	Gln	Val	Ser	Leu	Arg		500	505	510
	Thr	Arg	Phe	Gly	Met	His	Phe	Cys	Gly	Gly	Thr	Leu	Ile	Ser	Pro	Glu		515	520	525
15	Trp	Val	Leu	Thr	Ala	Ala	His	Cys	Leu	Glu	Lys	Ser	Pro	Arg	Pro	Ser		530	535	540
20	Ser	Tyr	Lys	Val	Ile	Leu	Gly	Ala	His	Gln	Glu	Val	Asn	Leu	Glu	Pro	545	550	555	560
25	His	Val	Gln	Glu	Ile	Glu	Val	Ser	Arg	Leu	Phe	Leu	Glu	Pro	Thr	Arg		565	570	575
	Lys	Asp	Ile	Ala	Leu	Leu	Lys	Leu	Ser	Ser	Pro	Ala	Val	Ile	Thr	Asp		580	585	590
30	Lys	Val	Ile	Pro	Ala	Cys	Leu	Pro	Ser	Pro	Asn	Tyr	Val	Val	Ala	Asp		595	600	605
35	Arg	Thr	Glu	Cys	Phe	Ile	Thr	Gly	Trp	Gly	Glu	Thr	Gln	Gly	Thr	Phe	610	615	620	
40	Gly	Ala	Gly	Leu	Leu	Lys	Glu	Ala	Gln	Leu	Pro	Val	Ile	Glu	Asn	Lys	625	630	635	640
	Val	Cys	Asn	Arg	Tyr	Glu	Phe	Leu	Asn	Gly	Arg	Val	Gln	Ser	Thr	Glu		645	650	655
45	Leu	Cys	Ala	Gly	His	Leu	Ala	Gly	Gly	Thr	Asp	Ser	Cys	Gln	Gly	Asp		660	665	670
50	Ser	Gly	Gly	Pro	Leu	Val	Cys	Phe	Glu	Lys	Asp	Lys	Tyr	Ile	Leu	Gln		675	680	685
	Gly	Val	Thr	Ser	Trp	Gly	Leu	Gly	Cys	Ala	Arg	Pro	Asn	Lys	Pro	Gly	690	695	700	
55	Val	Tyr	Val	Arg	Val	Ser	Arg	Phe	Val	Thr	Trp	Ile	Glu	Gly	Val	Met				

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705
710
715
720

5 Arg Asn Asn

<210> 7
<211> 714
<212> PRT
10 <213> Homo sapiens

<400> 7

15 Lys Val Tyr Leu Ser Glu Cys Lys Thr Gly Asn Gly Lys Asn Tyr Arg
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Gly Thr Met Ser Lys Thr Lys Asn Gly Ile Thr Cys Gln Lys Trp Ser
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Ser Thr Ser Pro His Arg Pro Arg Phe Ser Pro Ala Thr His Pro Ser
35 40 45

Glu Gly Leu Glu Glu Asn Tyr Cys Arg Asn Pro Asp Asn Asp Pro Gln
50 55 60

Gly Pro Trp Cys Tyr Thr Thr Asp Pro Glu Lys Arg Tyr Asp Tyr Cys
65 70 75 80

Asp Ile Leu Glu Cys Glu Glu Glu Cys Met His Cys Ser Gly Glu Asn
35 85 90 95

Tyr Asp Gly Lys Ile Ser Lys Thr Met Ser Gly Leu Glu Cys Gln Ala
100 105 110

Trp Asp Ser Gln Ser Pro His Ala His Gly Tyr Ile Pro Ser Lys Phe
115 120 125

Pro Asn Lys Asn Leu Lys Lys Asn Tyr Cys Arg Asn Pro Asp Arg Glu
130 135 140

Leu Arg Pro Trp Cys Phe Thr Thr Asp Pro Asn Lys Arg Trp Glu Leu
50 145 150 155 160

Cys Asp Ile Pro Arg Cys Thr Thr Pro Pro Pro Ser Ser Gly Pro Thr
165 170 175

55 Tyr Gln Cys Leu Lys Gly Thr Gly Glu Asn Tyr Arg Gly Asn Val Ala
180 185 190

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	Val	Thr	Val	Ser	Gly	His	Thr	Cys	Gln	His	Trp	Ser	Ala	Gln	Thr	Pro	
			195					200					205				
5	His	Thr	His	Asn	Arg	Thr	Pro	Glu	Asn	Phe	Pro	Cys	Lys	Asn	Leu	Asp	
		210					215					220					
10	Glu	Asn	Tyr	Cys	Arg	Asn	Pro	Asp	Gly	Lys	Arg	Ala	Pro	Trp	Cys	His	
	225					230					235					240	
15	Thr	Thr	Asn	Ser	Gln	Val	Arg	Trp	Glu	Tyr	Cys	Lys	Ile	Pro	Ser	Cys	
					245					250					255		
20	Asp	Ser	Ser	Pro	Val	Ser	Thr	Glu	Gln	Leu	Ala	Pro	Thr	Ala	Pro	Pro	
				260					265					270			
25	Glu	Leu	Thr	Pro	Val	Val	Gln	Asp	Cys	Tyr	His	Gly	Asp	Gly	Gln	Ser	
			275					280					285				
30	Tyr	Arg	Gly	Thr	Ser	Ser	Thr	Thr	Thr	Thr	Gly	Lys	Lys	Cys	Gln	Ser	
		290					295					300					
35	Trp	Ser	Ser	Met	Thr	Pro	His	Arg	His	Gln	Lys	Thr	Pro	Glu	Asn	Tyr	
	305					310					315					320	
40	Pro	Asn	Ala	Gly	Leu	Thr	Met	Asn	Tyr	Cys	Arg	Asn	Pro	Asp	Ala	Asp	
					325					330					335		
45	Lys	Gly	Pro	Trp	Cys	Phe	Thr	Thr	Asp	Pro	Ser	Val	Arg	Trp	Glu	Tyr	
				340					345					350			
50	Cys	Asn	Leu	Lys	Lys	Cys	Ser	Gly	Thr	Glu	Ala	Ser	Val	Val	Ala	Pro	
			355					360					365				
55	Pro	Pro	Val	Val	Leu	Leu	Pro	Asp	Val	Glu	Thr	Pro	Ser	Glu	Glu	Asp	
		370					375					380					
60	Cys	Met	Phe	Gly	Asn	Gly	Lys	Gly	Tyr	Arg	Gly	Lys	Arg	Ala	Thr	Thr	
	385				390						395					400	
65	Val	Thr	Gly	Thr	Pro	Cys	Gln	Asp	Trp	Ala	Ala	Gln	Glu	Pro	His	Arg	
					405					410					415		
70	His	Ser	Ile	Phe	Thr	Pro	Glu	Thr	Asn	Pro	Arg	Ala	Gly	Leu	Glu	Lys	
				420					425					430			

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	Asn	Tyr	Cys	Arg	Asn	Pro	Asp	Gly	Asp	Val	Gly	Gly	Pro	Trp	Cys	Tyr	
			435					440					445				
5	Thr	Thr	Asn	Pro	Arg	Lys	Leu	Tyr	Asp	Tyr	Cys	Asp	Val	Pro	Gln	Cys	
		450					455					460					
10	Ala	Ala	Pro	Ser	Phe	Asp	Cys	Gly	Lys	Pro	Gln	Val	Glu	Pro	Lys	Lys	
	465					470					475					480	
15	Cys	Pro	Gly	Arg	Val	Val	Gly	Gly	Cys	Val	Ala	His	Pro	His	Ser	Trp	
				485						490					495		
20	Pro	Trp	Gln	Val	Ser	Leu	Arg	Thr	Arg	Phe	Gly	Met	His	Phe	Cys	Gly	
				500					505					510			
25	Gly	Thr	Leu	Ile	Ser	Pro	Glu	Trp	Val	Leu	Thr	Ala	Ala	His	Cys	Leu	
			515					520					525				
30	Glu	Lys	Ser	Pro	Arg	Pro	Ser	Ser	Tyr	Lys	Val	Ile	Leu	Gly	Ala	His	
	530						535					540					
35	Gln	Glu	Val	Asn	Leu	Glu	Pro	His	Val	Gln	Glu	Ile	Glu	Val	Ser	Arg	
	545					550					555					560	
40	Leu	Phe	Leu	Glu	Pro	Thr	Arg	Lys	Asp	Ile	Ala	Leu	Leu	Lys	Leu	Ser	
					565					570					575		
45	Ser	Pro	Ala	Val	Ile	Thr	Asp	Lys	Val	Ile	Pro	Ala	Cys	Leu	Pro	Ser	
				580					585					590			
50	Pro	Asn	Tyr	Val	Val	Ala	Asp	Arg	Thr	Glu	Cys	Phe	Ile	Thr	Gly	Trp	
			595					600					605				
55	Gly	Glu	Thr	Gln	Gly	Thr	Phe	Gly	Ala	Gly	Leu	Leu	Lys	Glu	Ala	Gln	
		610					615					620					
60	Leu	Pro	Val	Ile	Glu	Asn	Lys	Val	Cys	Asn	Arg	Tyr	Glu	Phe	Leu	Asn	
	625					630					635					640	
65	Gly	Arg	Val	Gln	Ser	Thr	Glu	Leu	Cys	Ala	Gly	His	Leu	Ala	Gly	Gly	
				645						650					655		
70	Thr	Asp	Ser	Cys	Gln	Gly	Asp	Ser	Gly	Gly	Pro	Leu	Val	Cys	Phe	Glu	
				660					665					670			
75	Lys	Asp	Lys	Tyr	Ile	Leu	Gln	Gly	Val	Thr	Ser	Trp	Gly	Leu	Gly	Cys	

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	675	680	685
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	690	695	700
10	Thr Trp Ile Glu Gly Val Met Arg Asn Asn		
	705	710	
	<210> 8		
	<211> 713		
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	<213> Homo sapiens		
15	<400> 8		
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25	Thr Met Ser Lys Thr Lys Asn Gly Ile Thr Cys Gln Lys Trp Ser Ser		
		20	25 30
30	Thr Ser Pro His Arg Pro Arg Phe Ser Pro Ala Thr His Pro Ser Glu		
		35	40 45
35	Gly Leu Glu Glu Asn Tyr Cys Arg Asn Pro Asp Asn Asp Pro Gln Gly		
		50	55 60
40	Pro Trp Cys Tyr Thr Thr Asp Pro Glu Lys Arg Tyr Asp Tyr Cys Asp		
		65	70 75 80
45	Ile Leu Glu Cys Glu Glu Glu Cys Met His Cys Ser Gly Glu Asn Tyr		
		85	90 95
50	Asp Gly Lys Ile Ser Lys Thr Met Ser Gly Leu Glu Cys Gln Ala Trp		
		100	105 110
55	Asp Ser Gln Ser Pro His Ala His Gly Tyr Ile Pro Ser Lys Phe Pro		
		115	120 125
60	Asn Lys Asn Leu Lys Lys Asn Tyr Cys Arg Asn Pro Asp Arg Glu Leu		
		130	135 140
65	Arg Pro Trp Cys Phe Thr Thr Asp Pro Asn Lys Arg Trp Glu Leu Cys		
		145	150 155 160
70	Asp Ile Pro Arg Cys Thr Thr Pro Pro Pro Ser Ser Gly Pro Thr Tyr		
		165	170 175

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	Gln	Cys	Leu	Lys	Gly	Thr	Gly	Glu	Asn	Tyr	Arg	Gly	Asn	Val	Ala	Val	
				180					185					190			
5	Thr	Val	Ser	Gly	His	Thr	Cys	Gln	His	Trp	Ser	Ala	Gln	Thr	Pro	His	
			195					200					205				
10	Thr	His	Asn	Arg	Thr	Pro	Glu	Asn	Phe	Pro	Cys	Lys	Asn	Leu	Asp	Glu	
		210					215					220					
15	Asn	Tyr	Cys	Arg	Asn	Pro	Asp	Gly	Lys	Arg	Ala	Pro	Trp	Cys	His	Thr	
	225					230					235					240	
20	Thr	Asn	Ser	Gln	Val	Arg	Trp	Glu	Tyr	Cys	Lys	Ile	Pro	Ser	Cys	Asp	
				245						250					255		
25	Ser	Ser	Pro	Val	Ser	Thr	Glu	Gln	Leu	Ala	Pro	Thr	Ala	Pro	Pro	Glu	
				260					265					270			
30	Leu	Thr	Pro	Val	Val	Gln	Asp	Cys	Tyr	His	Gly	Asp	Gly	Gln	Ser	Tyr	
			275					280					285				
35	Arg	Gly	Thr	Ser	Ser	Thr	Thr	Thr	Thr	Gly	Lys	Lys	Cys	Gln	Ser	Trp	
		290					295					300					
40	Ser	Ser	Met	Thr	Pro	His	Arg	His	Gln	Lys	Thr	Pro	Glu	Asn	Tyr	Pro	
	305					310					315					320	
45	Asn	Ala	Gly	Leu	Thr	Met	Asn	Tyr	Cys	Arg	Asn	Pro	Asp	Ala	Asp	Lys	
				325						330					335		
50	Gly	Pro	Trp	Cys	Phe	Thr	Thr	Asp	Pro	Ser	Val	Arg	Trp	Glu	Tyr	Cys	
				340					345					350			
55	Asn	Leu	Lys	Lys	Cys	Ser	Gly	Thr	Glu	Ala	Ser	Val	Val	Ala	Pro	Pro	
		355						360					365				
60	Pro	Val	Val	Leu	Leu	Pro	Asp	Val	Glu	Thr	Pro	Ser	Glu	Glu	Asp	Cys	
		370					375					380					
65	Met	Phe	Gly	Asn	Gly	Lys	Gly	Tyr	Arg	Gly	Lys	Arg	Ala	Thr	Thr	Val	
	385					390					395					400	
70	Thr	Gly	Thr	Pro	Cys	Gln	Asp	Trp	Ala	Ala	Gln	Glu	Pro	His	Arg	His	
				405						410					415		

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	Ser	Ile	Phe	Thr	Pro	Glu	Thr	Asn	Pro	Arg	Ala	Gly	Leu	Glu	Lys	Asn	
				420					425					430			
5	Tyr	Cys	Arg	Asn	Pro	Asp	Gly	Asp	Val	Gly	Gly	Pro	Trp	Cys	Tyr	Thr	
			435					440					445				
10	Thr	Asn	Pro	Arg	Lys	Leu	Tyr	Asp	Tyr	Cys	Asp	Val	Pro	Gln	Cys	Ala	
		450					455					460					
15	Ala	Pro	Ser	Phe	Asp	Cys	Gly	Lys	Pro	Gln	Val	Glu	Pro	Lys	Lys	Cys	
	465					470					475					480	
20	Pro	Gly	Arg	Val	Val	Gly	Gly	Cys	Val	Ala	His	Pro	His	Ser	Trp	Pro	
				485						490					495		
25	Trp	Gln	Val	Ser	Leu	Arg	Thr	Arg	Phe	Gly	Met	His	Phe	Cys	Gly	Gly	
				500					505					510			
30	Thr	Leu	Ile	Ser	Pro	Glu	Trp	Val	Leu	Thr	Ala	Ala	His	Cys	Leu	Glu	
			515					520					525				
35	Lys	Ser	Pro	Arg	Pro	Ser	Ser	Tyr	Lys	Val	Ile	Leu	Gly	Ala	His	Gln	
		530					535					540					
40	Glu	Val	Asn	Leu	Glu	Pro	His	Val	Gln	Glu	Ile	Glu	Val	Ser	Arg	Leu	
	545					550					555					560	
45	Phe	Leu	Glu	Pro	Thr	Arg	Lys	Asp	Ile	Ala	Leu	Leu	Lys	Leu	Ser	Ser	
					565					570					575		
50	Pro	Ala	Val	Ile	Thr	Asp	Lys	Val	Ile	Pro	Ala	Cys	Leu	Pro	Ser	Pro	
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55	Asn	Tyr	Val	Val	Ala	Asp	Arg	Thr	Glu	Cys	Phe	Ile	Thr	Gly	Trp	Gly	
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60	Glu	Thr	Gln	Gly	Thr	Phe	Gly	Ala	Gly	Leu	Leu	Lys	Glu	Ala	Gln	Leu	
		610					615					620					
65	Pro	Val	Ile	Glu	Asn	Lys	Val	Cys	Asn	Arg	Tyr	Glu	Phe	Leu	Asn	Gly	
	625					630					635					640	
70	Arg	Val	Gln	Ser	Thr	Glu	Leu	Cys	Ala	Gly	His	Leu	Ala	Gly	Gly	Thr	
					645					650					655		
75	Asp	Ser	Cys	Gln	Gly	Asp	Ser	Gly	Gly	Pro	Leu	Val	Cys	Phe	Glu	Lys	

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5      Asp Lys Tyr Ile Leu Gln Gly Val Thr Ser Trp Gly Leu Gly Cys Ala
      675                                680                                685

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20     <220>
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      plasminogen

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35     Ser Thr Ser Pro His Arg Pro Arg Phe Ser Pro Ala Thr His Pro Ser
      35          40          45

40     Glu Gly Leu Glu Glu Asn Tyr Cys Arg Asn Pro Asp Asn Asp Pro Gln
      50          55          60

45     Gly Pro Trp Cys Tyr Thr Thr Asp Pro Glu Lys Arg Tyr Asp Tyr Cys
      65          70          75          80

50     Asp Val Pro Gln Cys Ala Ala Pro Ser Phe Asp Cys Gly Lys Pro Gln
      85          90          95

55     Val Glu Pro Lys Lys Cys Pro Gly Arg Val Val Gly Gly Cys Val Ala
      100         105         110

      His Pro His Ser Trp Pro Trp Gln Val Ser Leu Arg Thr Arg Phe Gly
      115         120         125

55     Met His Phe Cys Gly Gly Thr Leu Ile Ser Pro Glu Trp Val Leu Thr
      130         135         140

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5	Ile	Leu	Gly	Ala	His	Gln	Glu	Val	Asn	Leu	Glu	Pro	His	Val	Gln	Glu	
					165					170					175		
10	Ile	Glu	Val	Ser	Arg	Leu	Phe	Leu	Glu	Pro	Thr	Arg	Lys	Asp	Ile	Ala	
				180					185					190			
15	Leu	Leu	Lys	Leu	Ser	Ser	Pro	Ala	Val	Ile	Thr	Asp	Lys	Val	Ile	Pro	
			195					200					205				
20	Ala	Cys	Leu	Pro	Ser	Pro	Asn	Tyr	Val	Val	Ala	Asp	Arg	Thr	Glu	Cys	
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25	Phe	Ile	Thr	Gly	Trp	Gly	Glu	Thr	Gln	Gly	Thr	Phe	Gly	Ala	Gly	Leu	
	225					230					235					240	
30	Leu	Lys	Glu	Ala	Gln	Leu	Pro	Val	Ile	Glu	Asn	Lys	Val	Cys	Asn	Arg	
					245					250					255		
35	Tyr	Glu	Phe	Leu	Asn	Gly	Arg	Val	Gln	Ser	Thr	Glu	Leu	Cys	Ala	Gly	
				260					265					270			
40	His	Leu	Ala	Gly	Gly	Thr	Asp	Ser	Cys	Gln	Gly	Asp	Ser	Gly	Gly	Pro	
			275					280					285				
45	Leu	Val	Cys	Phe	Glu	Lys	Asp	Lys	Tyr	Ile	Leu	Gln	Gly	Val	Thr	Ser	
		290					295					300					
50	Trp	Gly	Leu	Gly	Cys	Ala	Arg	Pro	Asn	Lys	Pro	Gly	Val	Tyr	Val	Arg	
	305					310					315					320	
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Val Tyr Leu Ser Glu Cys Lys Thr Gly Asn Gly Lys Asn Tyr Arg Gly

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5	Thr	Met	Ser	Lys	Thr	Lys	Asn	Gly	Ile	Thr	Cys	Gln	Lys	Trp	Ser	Ser
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10	Thr	Ser	Pro	His	Arg	Pro	Arg	Phe	Ser	Pro	Ala	Thr	His	Pro	Ser	Glu
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15	Gly	Leu	Glu	Glu	Asn	Tyr	Cys	Arg	Asn	Pro	Asp	Asn	Asp	Pro	Gln	Gly
	50						55					60				
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25	Val	Pro	Gln	Cys	Ala	Ala	Pro	Ser	Phe	Asp	Cys	Gly	Lys	Pro	Gln	Val
					85					90					95	
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55	Glu	Val	Ser	Arg	Leu	Phe	Leu	Glu	Pro	Thr	Arg	Lys	Asp	Ile	Ala	Leu
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60	Leu	Lys	Leu	Ser	Ser	Pro	Ala	Val	Ile	Thr	Asp	Lys	Val	Ile	Pro	Ala
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75	Lys	Glu	Ala	Gln	Leu	Pro	Val	Ile	Glu	Asn	Lys	Val	Cys	Asn	Arg	Tyr
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	Glu	Phe	Leu	Asn	Gly	Arg	Val	Gln	Ser	Thr	Glu	Leu	Cys	Ala	Gly	His	
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5	Leu	Ala	Gly	Gly	Thr	Asp	Ser	Cys	Gln	Gly	Asp	Ser	Gly	Gly	Pro	Leu	
			275					280					285				
10	Val	Cys	Phe	Glu	Lys	Asp	Lys	Tyr	Ile	Leu	Gln	Gly	Val	Thr	Ser	Trp	
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15	Gly	Leu	Gly	Cys	Ala	Arg	Pro	Asn	Lys	Pro	Gly	Val	Tyr	Val	Arg	Val	
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35	Lys	Asn	Gly	Ile	Thr	Cys	Gln	Lys	Trp	Ser	Ser	Thr	Ser	Pro	His	Arg	
				20					25					30			
40	Pro	Arg	Phe	Ser	Pro	Ala	Thr	His	Pro	Ser	Glu	Gly	Leu	Glu	Glu	Asn	
			35					40					45				
45	Tyr	Cys	Arg	Asn	Pro	Asp	Asn	Asp	Pro	Gln	Gly	Pro	Trp	Cys	Tyr	Thr	
		50					55					60					
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55	<220>																
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      20          25          30

10     His Gly Tyr Ile Pro Ser Lys Phe Pro Asn Lys Asn Leu Lys Lys Asn
      35          40          45

15     Tyr Cys Arg Asn Pro Asp Arg Glu Leu Arg Pro Trp Cys Phe Thr Thr
      50          55          60

20     Asp Pro Asn Lys Arg Trp Glu Leu Cys Asp Ile Pro Arg Cys
      65          70          75

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25 <213> Homo sapiens

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      20          25          30

40     His Asn Arg Thr Pro Glu Asn Phe Pro Cys Lys Asn Leu Asp Glu Asn
      35          40          45

45     Tyr Cys Arg Asn Pro Asp Gly Lys Arg Ala Pro Trp Cys His Thr Thr
      50          55          60

50     Asn Ser Gln Val Arg Trp Glu Tyr Cys Lys Ile Pro Ser Cys
      65          70          75

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35 40 45

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20 25 30

45 His Ser Ile Phe Thr Pro Glu Thr Asn Pro Arg Ala Gly Leu Glu Lys
35 40 45

Asn Tyr Cys Arg Asn Pro Asp Gly Asp Val Gly Gly Pro Trp Cys Tyr
50 55 60

50 Thr Thr Asn Pro Arg Lys Leu Tyr Asp Tyr Cys Asp Val Pro Gln Cys
65 70 75 80

55 Claims

1. A polynucleotide comprising a nucleotide sequence encoding a polypeptide that is at least 95% identical to SEQ ID No. 2, having

- a) a single N-terminal kringle domain homologous to a kringle domain of native human plasminogen, wherein the last four amino acid residues within the kringle domain are V,P,Q, and C; and
- b) a C-terminal domain activation site and serine protease domain homologous to the corresponding domains in human plasminogen; wherein the polypeptide binds to immobilized lysine.

2. The polynucleotide of claim 1, wherein the encoded polypeptide is the sequence shown in SEQ ID NO: 2.
3. The polynucleotide of claim 1, wherein the nucleotide sequence of the polynucleotide is the sequence shown in SEQ ID NO: 1, or a degenerate variant thereof.
4. The polynucleotide of claim 1, wherein the polypeptide exhibits a lower binding affinity for fibrinogen than the binding affinity for fibrinogen of mini-plasmin.
5. The polynucleotide of claim 1, wherein the polypeptide exhibits higher binding affinity for partially cleaved fibrin than the binding affinity for partially cleaved fibrin of mini-plasmin.
6. The polynucleotide of Claim 1, wherein the polypeptide has reduced immunogenicity as compared to a reference polypeptide, wherein the reference polypeptide has a primary amino acid sequence identical to the primary amino acid sequence of the polypeptide with the proviso that the last four amino acid residues of the single N-terminal kringle domain of the reference polypeptide is not V,P,Q, and C.
7. A polypeptide encoded by the polynucleotide according to any one of claims 1 to 6.
8. The polypeptide of claim 7, wherein the polypeptide exhibits a fibrinolytic activity that is inhibited by α_2 -antiplasmin at a rate of inhibition that is at least about 5-fold faster than the rate of inhibition of the fibrinolytic activity of mini-plasmin by α_2 -antiplasmin.
9. The polypeptide of Claim 7, wherein the polypeptide has an amino acid sequence as shown in SEQ ID NO: 2, and conservative substitutions thereof.
10. The polypeptide of Claim 7, wherein the polypeptide has an arginine residue at a relative position analogous to that of position 85 of the amino acid sequence shown in SEQ ID NO: 2.
11. An expression vector comprising the polynucleotide of claim 1.
12. A cultured cell comprising the expression vector of claim 11.
13. A method for making one or more recombinant plasmin polypeptides having different N-termini, the method comprising:
 - a) providing a polypeptide according to claim 7
 - b) contacting the polypeptide provided in step a) with a protease under conditions sufficient to cleave one or more peptide bonds thereby forming one or more recombinant plasmin polypeptides having different N-termini.
14. The method of Claim 13, wherein the polypeptide has an amino acid sequence shown in SEQ ID NO: 2.
15. The method of Claim 13, wherein providing comprises expressing a DNA sequence shown in SEQ ID NO: 1, or a degenerate variant thereof, in *E. Coli*.

Patentansprüche

1. Polynukleotid umfassend eine Nukleotidsequenz, die für ein Polypeptid kodiert, das mindestens zu 95% identisch mit SEQ ID No. 2 ist, welches hat
 - a) eine einzelne N-terminale Kringle-Domäne homolog zu einer Kringle-Domäne von nativem menschlichen Plasminogen, wobei die letzten vier Aminosäurereste in der Kringle-Domäne V, P, Q und C sind; und
 - b) eine C-terminale Domänenaktivierungsseite und Serinproteasedomäne homolog zu den entsprechenden

Domänen in menschlichem Plasminogen; wobei das Polypeptid an immobilisiertes Lysin bindet.

2. Polynukleotid gemäß Anspruch 1, wobei das kodierte Polypeptid die Sequenz wie in SEQ ID NO: 2 gezeigt ist.

5 3. Polynukleotid gemäß Anspruch 1, wobei die Nukleotidsequenz des Polynukleotids die Sequenz wie in SEQ ID NO: 1 gezeigt ist, oder eine degenerierte Variante davon.

4. Polynukleotid gemäß Anspruch 1, wobei das Polynukleotid eine niedrigere Bindungsaffinität für Fibrinogen aufweist als die Bindungsaffinität für Fibrinogen von mini-Plasmin.

10 5. Polynukleotid gemäß Anspruch 1, wobei das Polynukleotid eine niedrigere Bindungsaffinität für teilweise gespaltenes Fibrinogen aufweist als die Bindungsaffinität für teilweise gespaltenes Fibrinogen von mini-Plasmin.

15 6. Polynukleotid gemäß Anspruch 1, wobei das Polypeptid reduzierte Immunogenizität hat verglichen mit einem Referenzpolypeptid, wobei das Referenzpolypeptid eine primäre Aminosäuresequenz hat, die identisch zu der primären Aminosäuresequenz des Polypeptids ist unter der Voraussetzung, dass die letzten vier Aminosäurereste der einzelnen N-terminalen Kringle-Domäne des Referenzpolypeptids nicht V, P, Q und C ist.

20 7. Polypeptid kodiert durch das Polynukleotid gemäß einem der Ansprüche 1 bis 6.

8. Polypeptid gemäß Anspruch 7, wobei das Polypeptid eine fibrinolytische Aktivität aufweist, die durch α_2 -Antiplasmin bei einer Inhibitions-
geschwindigkeit inhibiert wird, die wenigstens ungefähr 5-mal schneller als die Inhibitions-
geschwindigkeit der fibrinolytischen Aktivität von mini-Plasmin durch α_2 -Antiplasmin ist.

25 9. Polypeptid gemäß Anspruch 7, wobei das Polypeptid eine Aminosäuresequenz wie in SEQ ID NO: 2 gezeigt hat, und konservative Substitutionen davon.

30 10. Polypeptid gemäß Anspruch 7, wobei das Polypeptid einen Argininrest an einer relativen Position analog zu Position 85 der Aminosäuresequenz wie in SEQ ID NO: 2 gezeigt hat.

11. Expressionsvektor umfassend das Polynukleotid gemäß Anspruch 1.

12. Zellkultur umfassend den Expressionsvektor gemäß Anspruch 11.

35 13. Methode zum Herstellen eines oder mehrerer rekombinanten Plasminpolypeptide, welche unterschiedliche N-Termini haben, wobei die Methode umfasst:

40 a) Bereitstellen eines Polypeptids gemäß Anspruch 7
b) in Kontakt Bringen des Polypeptids wie in Schritt a) bereitgestellt mit einer Protease unter Bedingungen, die ausreichend sind, um eine oder mehrere Peptidbindungen zu spalten und dabei eine oder mehrere rekombinante Plasminpolypeptide zu bilden, welche unterschiedliche N-Termini haben.

14. Methode gemäß Anspruch 13, wobei das Polypeptid eine Aminosäuresequenz wie in SEQ ID NO: 2 gezeigt hat.

45 15. Methode gemäß Anspruch 13, wobei Bereitstellen umfasst Expressieren einer DNA-Sequenz wie in SEQ ID NO: 1 gezeigt, oder einer degenerierten Variante davon, in *E. Coli*.

Revendications

50 1. Polynucléotide comprenant une séquence nucléotidique codant pour un polypeptide qui est au moins identique à 95 % à SEQ ID NO : 2, comportant

55 a) un seul domaine kringle N-terminal homologue à un domaine kringle du plasminogène humain natif, dans lequel les quatre derniers résidus d'acides aminés au sein du domaine kringle sont V, P, Q et C ; et
b) un site d'activation de domaine C-terminal et un domaine de sérine protéase homologues aux domaines correspondants dans le plasminogène humain ; dans lequel le polypeptide se lie à une lysine immobilisée.

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2. Polynucléotide selon la revendication 1, dans lequel le polypeptide codé est la séquence représentée par SEQ ID NO : 2.
- 5 3. Polynucléotide selon la revendication 1, dans lequel la séquence nucléotidique du polynucléotide est la séquence représentée par SEQ ID NO : 1, ou l'un de ses variants dégénérés.
4. Polynucléotide selon la revendication 1, dans lequel le polypeptide présente une affinité de liaison inférieure pour le fibrinogène par rapport à l'affinité de liaison pour le fibrinogène de la mini-plasmine.
- 10 5. Polynucléotide selon la revendication 1, dans lequel le peptide présente une affinité de liaison supérieure pour la fibrine partiellement clivée par rapport à l'affinité de liaison pour la fibrine partiellement clivée de la mini-plasmine.
6. Polynucléotide selon la revendication 1, dans lequel le polypeptide présente une réduction de l'immunogénicité comparativement à un polypeptide de référence, dans lequel le polypeptide de référence a une séquence primaire d'acides aminés identique à la séquence primaire d'acides aminés du polypeptide à condition que les quatre derniers résidus d'acides aminés du seul domaine kringle N-terminal du polypeptide de référence ne soient pas V, P, Q et C.
- 15 7. Polypeptide codé par le polynucléotide selon l'une quelconque des revendications 1 à 6.
- 20 8. Polypeptide selon la revendication 7, dans lequel le polypeptide présente une activité fibrinolytique qui est inhibée par l' α_2 -antiplasmine à une vitesse d'inhibition qui est au moins environ 5 fois plus rapide que la vitesse d'inhibition de l'activité fibrinolytique de la mini-plasmine par l' α_2 -antiplasmine.
9. Polypeptide selon la revendication 7, dans lequel le polypeptide a une séquence d'acides aminés telle que représentée par SEQ ID NO : 2, et ses substitutions conservatrices.
- 25 10. Polypeptide selon la revendication 7, dans lequel le polypeptide a un résidu d'arginine au niveau d'une position relative analogue à celle de la position 85 de la séquence d'acides aminés représentée par SEQ ID NO : 2.
- 30 11. Vecteur d'expression comprenant le polynucléotide selon la revendication 1.
12. Cellule cultivée comprenant le vecteur d'expression selon la revendication 11.
- 35 13. Procédé de fabrication d'un ou de plusieurs polypeptides de plasmine recombinants ayant des extrémités N-terminales différentes, le procédé comprenant :
 - a) la fourniture d'un polypeptide selon la revendication 7
 - b) la mise en contact du polypeptide fourni dans l'étape a) avec une protéase dans des conditions suffisantes pour cliver une ou plusieurs liaisons peptidiques, formant de cette façon un ou plusieurs polypeptides de plasmine recombinants ayant des extrémités N-terminales différentes.
- 40 14. Procédé selon la revendication 13, dans lequel le polypeptide a une séquence d'acides aminés représentée par SEQ ID NO : 2.
- 45 15. Procédé selon la revendication 13, dans lequel la fourniture comprend l'expression d'une séquence d'ADN représentée par SEQ ID NO : 1, ou de l'un de ses variants dégénérés, dans *E. coli*.

50

55

FIG. 1

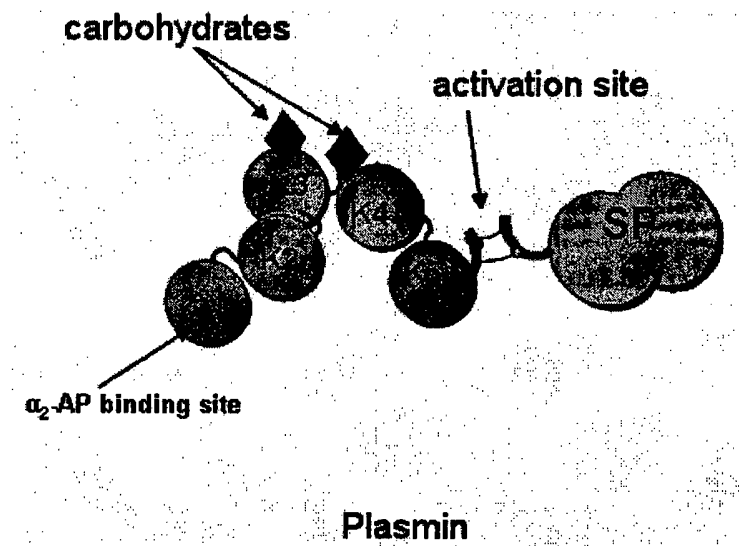


FIG. 2

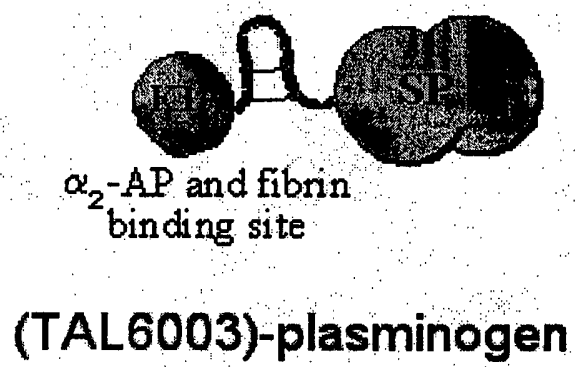


FIG. 3

-19 1
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78
YHSKEQQCVIMAENRKSSIIIR

136 143 153 162
ILECEEECMHCSGENYDG
kringle 1

234
KISKMSGLECAWDSQSPHAGYIPSKFPKNLKKNYCRNPDRELRPWCFTTDPNKRWELCDIP
kringle 2

RCTTPPPSSGPTYQCLKGTGENYRGNVAVTVSGHTCQHWSAQTPHTHNRTPENFPCKNLDENYCR
kringle 3

324
NPDGKRAPWCHTTNSQVRWEYCKIPSCDSSPVSTEQLAPTAPPELTPVVQDCYHGDGQSYRGTS

426
TTTTGKKCQSWSSMTPHRHQKTPENYPNAGLTMNYCRNPDADKGPWCFTTDPNKRWELCDIP
kringle 4

GTEASVVAPPPVLLPDVETPSEEDCMFGNGKGYRGKRATTVTGTGTPCQDWAAQEPHRHSIFTPET
kringle 5

532 542 561
NPRAGLEKNYCRNPDGDVGGPWCYTTPNPKLYDYCD

791
(SEQ ID NO:4)

FIG. 4

HK1	<u>CKTGNGKNYR</u>	<u>GTMSKTKNGI</u>	<u>TCQKWSSTSP</u>	<u>HR-PRFSPAT</u>	<u>HPSEGLEENY</u>
HK2	<u>CMHCSGENYD</u>	<u>GKISKTM SGL</u>	<u>ECQAWDSOSP</u>	<u>HA-HGYIPSK</u>	<u>FPNKNLKKNY</u>
HK3	<u>CLKGTGENYR</u>	<u>GNVAVTVSGH</u>	<u>TCQHWSAQTP</u>	<u>HT-HNRTPEN</u>	<u>FPCKNLDENY</u>
HK4	<u>CYHGDGQSYR</u>	<u>GTSSTTTTGK</u>	<u>KCQSWSSMTP</u>	<u>HR-HQKTPEN</u>	<u>YPNAGLTMNY</u>
HK5	<u>CMFGNGKGYR</u>	<u>GKRATTVTGT</u>	<u>PCQDWAAQEP</u>	<u>HRHSIFTPET</u>	<u>NPRAGLEKNY</u>

(con't)

HK1	<u>CRNPDNDPQG</u>	<u>PWCYT TDPEK</u>	<u>RYDYCDILEC</u>	(SEQ ID NO:11)
HK2	<u>CRNPDRE-LR</u>	<u>PWCFT TDPNK</u>	<u>RWELCDIPRC</u>	(SEQ ID NO:12)
HK3	<u>CRNPDGK-RA</u>	<u>PWCHTTNSQV</u>	<u>RWEYCKIPSC</u>	(SEQ ID NO:13)
HK4	<u>CRNPDAD-KG</u>	<u>PWCFT TDPSV</u>	<u>RWEYCNLKKC</u>	(SEQ ID NO:14)
HK5	<u>CRNPDGDVGG</u>	<u>PWCYT TNPRK</u>	<u>LYDYCDVPQC</u>	(SEQ ID NO:15)

FIG. 5

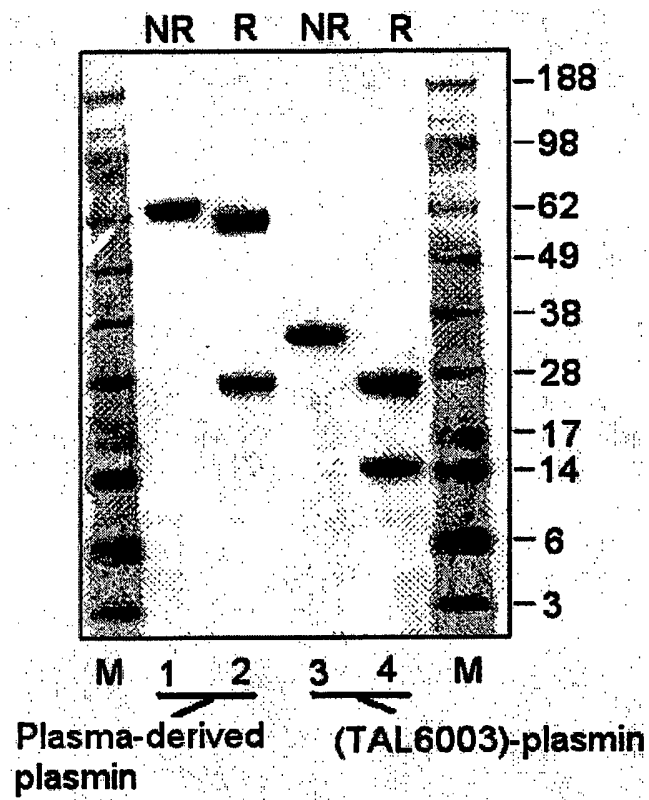


FIG. 6

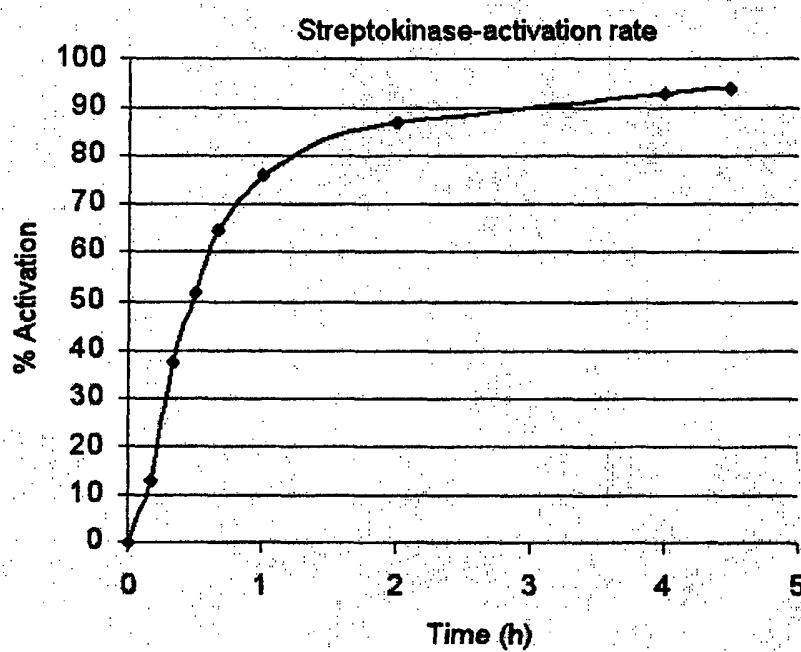


FIG. 7

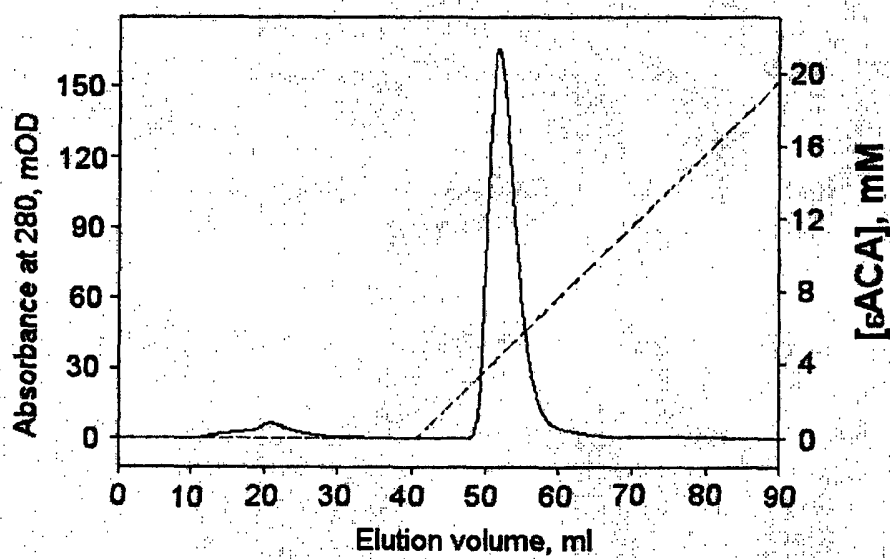


FIG. 8

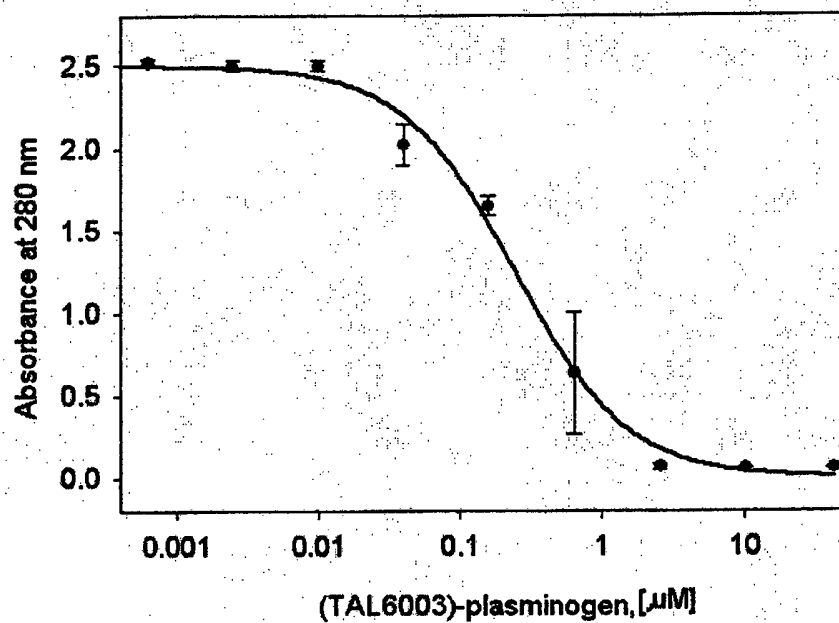


FIG. 9

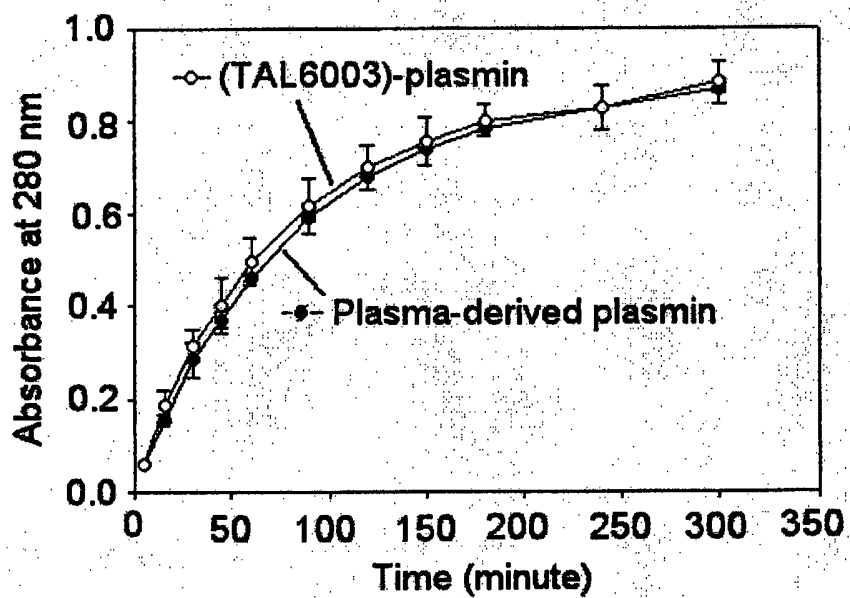
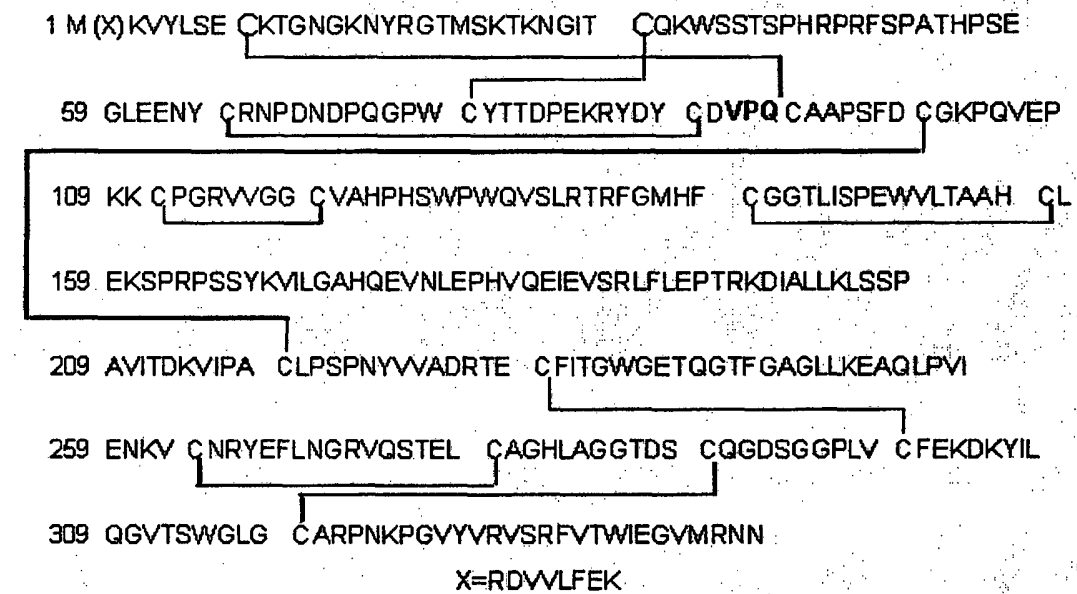


FIGURE 10



(SEQ ID NO:2)

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REKOMBINÁNSAN MÓDOSÍTOTT PLAZMIN

Szabadalmi igénypontok

1. Polinukleotid, amely tartalmaz olyan polipeptidet kódoló nukleotidszekvenciát, amely polipeptid legalább 95%-ban azonos a SEQ ID NO:2-vel (azaz a 2-es azonosító számú szekvenciával) és tartalmaz
 - a) egyetlen N-terminális kringle-domént, amely homológ a natív humán plazminogén egy kringle-doménjével, ahol a kringle-domén utolsó négy aminosavja V, P, Q és C; és
 - b) egy C-terminális domén aktivációs helyet és egy szerin-proteáz domént, amelyek homológok a humán plazminogén megfelelő doménjeivel; ahol a polipeptid kötődik (azaz képes kötődni) immobilizált lizinhez.
2. Az 1. igénypont szerinti polinukleotid, ahol a kódolt polipeptid a SEQ ID NO:2-nél bemutatott szekvencia.
3. Az 1. igénypont szerinti polinukleotid, ahol a polinukleotid nukleotidszekvenciája a SEQ ID NO:1-nél bemutatott szekvencia, vagy annak egy degenerált változata.
4. Az 1. igénypont szerinti polinukleotid, ahol polipeptidnek kisebb a fibrinogén iránti kötési affinitása, mint a mini-plazmin fibrinogén iránti kötési affinitása.
5. Az 1. igénypont szerinti polinukleotid, ahol polipeptidnek nagyobb a részlegesen elhasított fibrin iránti kötési affinitása, mint a mini-plazmin kötési affinitása a részlegesen elhasított fibrin iránt.

6. Az 1. igénypont szerinti polinukleotid, ahol egy referencia-polipeptiddel összehasonlítva a polipeptidnek kisebb az immunogenitása, ahol a referencia-polipeptid primer aminosav-szekvenciája azonos a polipeptid primer aminosav-szekvenciájával azzal a megkötéssel, hogy a referencia-polipeptid egyetlen N-terminális kringle-doménjének utolsó négy aminosavja nem V, P, Q és C.
7. Polipeptid, amelyet az 1-6. igénypontok bármelyike szerinti polinukleotid kódol.
8. A 7. igénypont szerinti polipeptid, ahol a polipeptid olyan fibrinolitikus aktivitást mutat, amelyet az $\alpha 2$ -anti-plazmin olyan gátlási sebességgel gátol, amely legalább körülbelül ötször gyorsabb, mint az a gátlási sebesség, amellyel az $\alpha 2$ -anti-plazmin a mini-plazmin fibrinolitikus aktivitását gátolja.
9. A 7. igénypont szerinti polinukleotid, ahol a polipeptid aminosav-szekvenciája a SEQ ID NO:2-nél bemutatott szekvencia, és annak konzervatív szubsztitúciói.
10. A 7. igénypont szerinti polipeptid, ahol a polipeptid a SEQ ID NO:2-nél bemutatott aminosav-szekvencia 85. pozíciójával analóg relatív pozícióban arginint tartalmaz.
11. Expressziós vektor, amely tartalmazza az 1. igénypont szerinti polinukleotidot.
12. Tenyésztett sejt, amely tartalmazza a 11. igénypont szerinti expressziós vektort.
13. Eljárás egy vagy több, eltérő N-terminálissal rendelkező rekombináns plazmin polipeptid előállítására, amely eljárás tartalmazza:
 - a) egy 7. igénypont szerinti polipeptid biztosítását

b) az a) lépésben biztosított polipeptid olyan körülmények között történő érintkeztetését egy proteázzal, amely elégséges egy vagy több peptidkötés elhasításához, amelynek révén egy vagy több, eltérő N-terminálissal rendelkező rekombináns plazmin polipeptid képződik.

14. A 13. igénypont szerinti eljárás, ahol a polipeptid aminosav-szekvenciája SEQ ID NO:2-nél bemutatott szekvencia.
15. A 13. igénypont szerinti eljárás, ahol a biztosítás azt jelenti, hogy *E. coli*-ban expresszáltunk egy SEQ ID NO:1 szerinti DNS-szekvenciát, vagy annak egy degenerált változatát.