

FACTOR B PROTEASES

CROSS REFERENCE TO RELATED APPLICATION

[0001] This application claims priority to U.S. Provisional Application No. 63/135,496 filed on January 8, 2021, and U.S. Provisional Application No. 63/221,108 filed on July 13, 2021, the contents of which are incorporated herein by reference in their entireties.

BACKGROUND

[0002] The complement system includes the classical, alternative, and lectin pathways, and is tightly controlled by a number of regulators and components. One such component is Complement Factor B (interchangeably referred to herein as CFB, Factor B, FB), a serine protease proenzyme that circulates in blood as a single chain polypeptide. When Factor B associates with the active forms of C3, such as surface-bound C3b or fluid-phase C3(H₂O), to form a proconvertase complex, Factor B can subsequently be cleaved by Factor D into two fragments, Ba and Bb. The cleavage site of Factor B targeted by Factor D includes an Arg234-Lys235 bond. The resulting Ba cleavage product is non-catalytic and released from the complex, while the resulting Bb cleavage product is a catalytic serine protease that can then cleave C3 into C3a and C3b. This generation of C3b is part of an amplification loop of the complement system, allowing C3b to bind to another Factor B to form C3bBb.

[0003] Factor B and its cleavage products regulate complement activation. Dysregulated complement has been implicated in diseases involving the complement system, and thus needed are methods for modulating or inhibiting particular points of regulation within the complement system, such as the generation of the inactive Factor B fragments. Provided herein are compositions and methods that address these needs.

SUMMARY

[0004] Provided herein are engineered, non-naturally occurring chymotrypsin-like serine proteases. Also provided herein are methods of making and using such non-naturally occurring chymotrypsin-like serine protease. The engineered proteases provided herein may be useful for treating a disease or condition associated with dysregulation of the complement system, or overactivation of complement.

[0005] Accordingly, in one aspect, provided herein is an engineered protease of the S1A serine protease family, wherein the engineered protease is specific for and is capable of cleaving Factor B. More specifically, the engineered protease of the disclosure comprises a modified chymase protease domain, a modified membrane type serine protease 1 (MTSP-1) protease domain, a modified urokinase-type plasminogen activator (uPA) protease domain, or a modified Kallikrein-related peptidase 5 (KLK5) protease domain, wherein the engineered protease is capable of cleaving Factor B. Modifications include one or more of a substitution, addition, and deletion of one or more amino acid residues, and/or one or more of a substitution, addition, and deletion of one or more domains of the chymotrypsin-like serine protease.

[0006] In some embodiments, the engineered protease is based on a MTSP-1 protease domain. In some embodiments, the engineered protease is not based on a MTSP-1 protease domain. In some embodiments, the engineered protease comprises one or more modifications with respect to a MTSP-1 protease domain comprising an amino acid sequence as set forth in SEQ ID NO: 7. In some embodiments, the one or more modifications are selected from those presented in Table 5A. In some embodiments, the one or more modifications are selected from those exemplary mutation strings presented in Table 5B. In some embodiments, the engineered protease comprises a modified membrane type serine protease 1 (MTSP-1) protease domain having at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 95% sequence identity to SEQ ID NO: 7.

[0007] In some embodiments, the engineered protease is based on a uPA protease domain. In some embodiments, the engineered protease is not based on a uPA protease domain. In some embodiments, the engineered protease comprises one or more modifications with respect to a uPA protease domain comprising an amino acid sequence as set forth in SEQ ID NO: 22. In some embodiments, the one or more modifications are selected from those presented in Table 3A. In some embodiments, the one or more modifications are selected from those exemplary mutation strings presented in Table 3B. In some embodiments, the engineered protease comprises a modified urokinase-type plasminogen activator (uPA) protease domain having at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at

least 96%, at least 97%, at least 98%, at least 99%, or at least 95% sequence identity to SEQ ID NO: 22.

[0008] In some embodiments, the engineered protease comprises one or more modifications with respect to a chymase protease domain comprising an amino acid sequence as set forth in SEQ ID NO: 6. In some embodiments, the one or more modifications are selected from those presented in Table 7A. In some embodiments, the one or more modifications are selected from those exemplary mutation strings presented in Table 7B. In some embodiments, the engineered protease comprises a modified chymase protease domain having at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 95% sequence identity to SEQ ID NO: 6.

[0009] In some embodiments, the engineered protease is based on KLK5 protease domain, optionally comprising one or more amino acid modifications of SEQ ID NO: 23. In some embodiments, the engineered protease comprises a modified Kallikrein-related peptidase 5 (KLK5) protease domain having at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 95% sequence identity to SEQ ID NO: 23.

[0010] In some embodiments, cleavage of Factor B by the engineered protease generates one or more functionally inactive fragments. In some embodiments, the one or more functionally inactive fragments are capable of reducing complement activation. In some embodiments, cleavage of Factor B results in the generation of a Factor B fragment that is reduced in function.

[0011] In some embodiments, the Factor B is a rodent Factor B. In some embodiments, the Factor B is a non-human primate Factor B. In some embodiments, the non-human primate is cynomolgus monkey. In some embodiments, the Factor B is human Factor B. In some embodiments, the Factor B comprises the amino acid sequence as set forth in SEQ ID NO: 1.

[0012] In some embodiments, cleavage of Factor B occurs at a site not targeted by Factor D. In some embodiments, cleavage at the site generates at least two fragments that are not Ba and

Bb. In some embodiments, cleavage at the site results in a reduction of the generation of Factor B cleavage products Ba and Bb as compared to cleavage by Factor D.

[0013] In some embodiments, cleavage of Factor B occurs at a site that is targeted by Factor D. In some embodiments, the site targeted by Factor D comprises QQKR/KIV (SEQ ID NO: 9). In some embodiments, the Factor B cleavage site comprises a sequence selected from: WEHR/KGT (SEQ ID NO: 10), KNQKR/QKQ (SEQ ID NO: 11), DVFY/QMI (SEQ ID NO: 12), EGVD/AE (SEQ ID NO: 13), DHKL/KSG (SEQ ID NO: 14), TPW/SLA (SEQ ID NO: 15), KVSEAD (SEQ ID NO: 20), IRPSKG (SEQ ID NO: 4), GGEKRD (SEQ ID NO: 5), GKKEAG (SEQ ID NO: 3), and DHKL/KSG (SEQ ID NO: 21).

[0014] In some embodiments, the engineered protease is based on MTSP-1 or uPA (includes a MTSP-1 or uPA protease domain) and the cleavage site in the Factor B comprises a sequence selected from: WEHR/KGT (SEQ ID NO: 10) and KNQKR/QKQ (SEQ ID NO: 11).

[0015] In some embodiments, the engineered protease is based on chymase protease domain. In some embodiments, the engineered protease is based on chymase protease domain, and the cleavage site comprises a sequence selected from DVFY/QMI (SEQ ID NO: 12), EGVD/AE (SEQ ID NO: 13), DHKL/KSG (SEQ ID NO: 14), and TPW/SLA (SEQ ID NO: 15).

[0016] In some embodiments, cleavage of Factor B results in the generation of a Factor B fragment that is reduced in function or results in a Factor B that is reduced in function. In some embodiments, the function of Factor B or a Factor B fragment is an interaction with at least one complement component. In some embodiments, the function of Factor B or a Factor B fragment is an interaction with hydrolyzed soluble C3. In some embodiments, the function of Factor B or a Factor B fragment is an interaction with C3b. In some embodiments, the function of Factor B or a Factor B fragment is an interaction with membrane-bound C3b.

[0017] In some embodiments, cleavage occurs when Factor B is not bound to C3b.

[0018] In some embodiments, the cleavage activity for a non-Factor B peptide substrate is about equal to or less than cleavage activity for the Factor B site.

[0019] In some embodiments, the engineered protease has a k_{cat}/K_m of about 100, about 200, about 300, about 400, about 500, about 600, about 700, about 800, about 900, about 1,000, about

1,000, about 1,100, about 1,200, about 1,300, about 1,400, about 1,500, about 1,600, about 1,700, about 1,800, or about 1,900 $\text{M}^{-1} \text{s}^{-1}$ for Factor B cleavage. In some embodiments, the engineered protease has a $k_{\text{cat}}/K_{\text{m}}$ of about 10^3 to about $10^9 \text{M}^{-1} \text{s}^{-1}$ for Factor B cleavage. In some embodiments, the engineered protease has an EC_{50} for Factor B of less than about 20 nM. In some embodiments, the engineered protease has an EC_{50} for Factor B of less than about 1 nM. In some embodiments, the engineered protease has an EC_{50} for Factor B of about 20, about 25, or about 60 nM. In some embodiments, the engineered protease has an EC_{50} for cleaving Factor B of about 1,000 to about 4,500 nM.

[0020] In some embodiments, the engineered protease has a plasma half-life in human plasma of over about 72 hours. In some embodiments, the engineered protease has a plasma half-life in human plasma of over about 120 hours. In some embodiments, the engineered protease has a plasma half-life in human plasma of about 7 days. In some embodiments, the catalytic activity is about 10% to about 50%, or about 90% to about 100%.

[0021] In some embodiments, the engineered protease has an increased half-life compared to an MTSP-1 protease domain that is not modified. In some embodiments, the engineered protease has an increased bioavailability compared to an MTSP-1 protease domain that is not modified. In some embodiments, the engineered protease has an increased half-life compared to a uPA protease domain that is not modified. In some embodiments, the engineered protease has an increased bioavailability compared to a uPA protease domain that is not modified. In some embodiments, the engineered protease has an increased half-life compared to a chymase that is not modified. In some embodiments, the engineered protease has an increased bioavailability compared to a chymase protease domain that is not modified. In some embodiments, the engineered protease has an increased bioavailability compared to a KLK5 protease domain that is not modified.

[0022] In some embodiments, the engineered protease is non-immunogenic.

[0023] In some embodiments, the engineered protease is in a zymogen form. In some embodiments, the engineered protease is in an active form.

[0024] In some embodiments, the engineered protease further comprises a half-life extender. Exemplary half-life extenders include Human Serum Albumin (HSA) and Fc (e.g., IgG1) fused to the engineered protease.

[0025] In another aspect, a method of inactivating Factor B is provided, comprising contacting the Factor B with any of the engineered proteases of the disclosure. In some embodiments, complement activation is inhibited. In some embodiments, the classical pathway of the complement pathway is inhibited. In some embodiments, the alternate pathway of the complement pathway is inhibited. In some embodiments, the lectin pathway of the complement pathway is inhibited.

[0026] In some embodiments, the method is *in vitro*. In some embodiments, the method is *in vivo*.

[0027] In another aspect, a method of treating a disease or condition in a subject in need thereof is provided, comprising administering to the subject any one of the engineered proteases of the disclosure. In some embodiments, the disease or condition is associated with dysregulated complement. In some embodiments, the disease or condition is an inflammatory disease or condition. In some embodiments, the treatment is a replacement therapy. In some embodiments, the treatment blocks complement activation. In some embodiments, the treatment modulates autoimmunity. In some embodiments, the disease or condition is a congenital complement deficiency. In some embodiments, the treatment is for endothelial or kidney cell injury.

[0028] In some embodiments, the disease or condition is selected from lupus nephritis, C3 glomerulopathy (C3G), primary IgA nephropathy, kidney transplant ischemia and reperfusion (I/R) injury, antineutrophil cytoplasmic antibody (ANCA)-associated vasculitides (AAV), atypical hemolytic uremic syndrome (aHUS), membranous nephropathy (MN) and paroxysmal nocturnal hemoglobinuria (PNH). In some embodiments, the disease or condition is a control protein deficiency. In some embodiments, the disease or condition is a secondary complement disorder. In some embodiments, the disease or condition is an immunity-related disease or condition.

[0029] In some embodiments, the engineered protease is administered to the subject subcutaneously.

[0030] In some embodiments, engineered protease is activated *in situ* at the site of a dysregulated complement component.

[0031] In some embodiments, the engineered protease is provided in a liquid stable formulation.

[0032] In another aspect, a pharmaceutical composition comprising any of the engineered proteases of the disclosure, and optionally a pharmaceutically acceptable carrier, is provided.

[0033] In some embodiments the engineered protease is provided in a liquid stable formulation.

[0034] In some embodiments the composition is formulated for subcutaneous administration.

BRIEF DESCRIPTION OF THE DRAWINGS

[0035] FIG. 1A depicts a schematic diagram of a naturally occurring Factor B, showing the site at which Factor B is cleaved by Factor D (FD cleavage site; SEQ ID NOs: 13, 9, 20, residues 1-6 of SEQ ID NO: 12, SEQ ID NOs: 4, 5, 3, and residues 1-6 of SEQ ID NO: 11).

[0036] FIG. 1B and FIG. 1C depict two views of the protein structure of Factor B showing various cleavage sites (SEQ ID NOs: 13, 9, 11, 10, residues 1-6 of SEQ ID NO: 12 and SEQ ID NO: 14).

[0037] FIG. 1D depicts a Coomassie gel showing examples of Factor B cleavage by chymase-based engineered proteases.

[0038] FIG. 1E depicts a graph showing examples of Factor B cleavage by two chymase-based engineered proteases having low EC₅₀.

[0039] FIG. 2A and FIG. 2B depict schematic diagrams of a pro-chymase and a mature chymase, respectively. The mature chymase provides an exemplary scaffold of the disclosure.

[0040] FIG. 3A and FIG. 3B depict schematic diagrams of the extracellular portion of Membrane Type Serine Protease 1 (MTSP-1) and the serine protease domain of MTSP-1, respectively.

[0041] FIG. 3C and FIG. 3D depict schematic diagrams of urokinase-type plasminogen activator (uPA or u-PA).

[0042] FIG. 4A, FIG. 4B, FIG. 4C, FIG. 4D, and FIG. 4E depict graphs showing the stability of the engineered chymase-based engineered proteases tested using a peptide substrate.

[0043] FIG. 5 depicts a bar graph of controls used for a Factor B add-back hemolysis assay.

[0044] FIG. 6 depicts a graph showing the standard curves of the results of the Factor B add-back hemolysis assay.

[0045] FIG. 7A depicts a graph showing hemolysis inhibition with KLK5 and a chymase-based engineered protease C22S/F173Y/D175N/A226R.

[0046] FIG. 7B depicts a graph showing FB cleavage with two concentrations of KLK5 and a chymase-based engineered protease C22S/F173Y/D175N/A226R.

[0047] FIG. 8A and FIG. 8B depict Coomassie gels showing examples of Factor B cleavage with KLK5 and a chymase-based engineered protease C22S/F173Y/D175N/A226R.

[0048] FIG. 9 depicts mass spectrometry (MS) data identifying the cleavage site at ²³⁴Arg within the QQKR/KIV (SEQ ID NO: 9) cleavage site of Factor B by KLK5, and identifying the cleavage site at ²²¹Asp within the EGVDAE (SEQ ID NO: 13) cleavage site of Factor B by the chymase-based engineered protease C22S/A226R.

[0049] FIG. 10 is a schematic depicting the general method for measuring complement activation and cytokine release from tissues of a mouse model of acute respiratory distress syndrome (ARDS) treated with a chymase-based engineered protease.

[0050] FIG. 11A, FIG. 11B, and FIG. 11C depict the pulmonary congestion index, body weight loss, and neutrophil to lymphocyte ratio in bronchoalveolar lavage fluid (BALF NLR), respectively, measured in a mouse model of acute respiratory distress syndrome (ARDS).

[0051] FIG. 12A, FIG. 12B, FIG. 12C, and FIG. 12D depict the results of BALF and lung cytokine measurement from mouse tissue after treatment with a chymase-based engineered protease.

[0052] FIG. 13 is a schematic depicting the general method for measuring lung function in a mouse model of acute respiratory distress syndrome (ARDS) treated with a chymase-based engineered protease of the disclosure.

[0053] FIG. 14 depicts the results of plethysmography measurements and significant protection to pulmonary congestion upon administration of one of the chymase-based engineered proteases of the disclosure.

[0054] FIG. 15 is a SDS-PAGE (reduced) depicting the expression, purification, and activation of a chymase-based engineered protease of the disclosure.

[0055] FIG. 16 is a SDS-PAGE depicting expression of chymase-based engineered proteases in HEK293 cells.

[0056] FIG. 17 is a SDS-PAGE depicting expression of chymase-based engineered proteases fused to human HSA or Fc in HEK293 cells.

DETAILED DESCRIPTION

[0057] The disclosure provides compositions and methods useful for modulating the signaling and regulation of the complement system. Specifically, provided herein are engineered proteases comprising protease domains of the chymotrypsin-like S1A serine protease family such engineered proteases of the disclosure are specific for, and capable of cleaving Factor B. These engineered proteases comprise modified protease domains and are generated using the methods and sequences provided herein.

[0058] The engineered proteases of the disclosure target Factor B for cleavage and are interchangeably referred to as “Complement Factor B degraders” or “CFB degraders”. Use of these engineered proteases may (1) result in the cleavage of Factor B into fragments that are neither Ba nor Bb, or may (2) result in the cleavage of Factor B into Ba and Bb but which are functionally inactive. Without being bound to any theory or mechanism, fragments Ba and Bb resulting from cleavage of Factor B while Factor B is not complexed with C3 or C3b, are thought to be functionally inactive fragments and may further reduce or inhibit complement activation, act to limit an increase complement activation, and/or limit/reduce the amplification of complement pathways. In either of these scenarios, these cleavage products may have a

function that is not a native function of Ba and Bb, or may be inactive fragments. As used herein, a “functionally inactive” fragment of Factor B refers to a fragment of Factor B, which is a cleavage product that may reduce complement activation, may limit an increase in complement activation, and/or may limit/reduce the amplification of the complement pathways.

[0059] In some embodiments, the engineered proteases target Factor B for cleavage at a cleavage site that is not targeted by Factor D. In other embodiments, the engineered proteases target Factor B at a site that is targeted by Factor D, before Factor B associates with and forms a complex with C3b, thus preventing the formation of the proconvertase complex.

[0060] The disclosure also provides methods of making and using such engineered proteases, for example, in treating a disease or condition associated with complement dysregulation, e.g. treating an overactive complement response.

Chymotrypsin-Like Serine Proteases Useful for Modulation of the Complement System by Targeting Factor B

[0061] Provided herein are engineered proteases comprising one or more modifications with respect to a naturally occurring chymotrypsin-like serine protease. As used herein, an “engineered” protease of the disclosure is a serine protease of the S1A family that is non-naturally occurring, and comprises one or more modifications with respect to a wild type or naturally occurring serine protease of the S1A family. As used herein, a “modification” to a naturally occurring chymotrypsin-like serine protease of the S1A family, includes one or more of: a deletion of one or more amino acid residues, a deletion of one or more domains, a substitution of one or more amino acid residues, an insertion of one or more amino acid residues, an insertion of one or more domains, and a substitution of one or more domains.

[0062] In some embodiments, the engineered proteases of the disclosure comprise a non-naturally occurring serine protease domain of the S1A family, which domain comprises one or more modifications with respect to a wild type or naturally occurring serine protease domain of the S1A family. As used herein, a “modification” to a naturally occurring chymotrypsin-like serine protease domain of the S1A family, includes one or more of a deletion of one or more

amino acid residues, a substitution of one or more amino acid residues, and an insertion of one or more amino acid residues.

[0063] The engineered proteases of the disclosure comprise a non-naturally occurring serine protease domain of the S1A family. In some embodiments, the engineered proteases of the disclosure comprise a non-naturally occurring serine protease domain of the S1A family and also comprise additional sequences and/or additional domains. In some embodiments, the engineered proteases of the disclosure consist of a non-naturally occurring serine protease domain of the S1A family. In some embodiments, the engineered proteases of the disclosure consist essentially of a non-naturally occurring serine protease domain of the S1A family, and may include additional sequences useful for expression, stability, improved pharmacokinetics, subcutaneous delivery, tissue targeting and the like.

[0064] It is noted that as used herein, a “naturally occurring chymotrypsin-like serine protease” of the S1A family refers to such protease that is present in nature, even if it is not the wild type sequence. Stated differently, the naturally occurring serine protease is not engineered. The naturally occurring serine protease may be with or without a signal sequence, and with or without an activation peptide, and may be of any species.

[0065] The engineered proteases provided herein are designed to cleave Factor B. In some embodiments, the engineered proteases provided herein target Factor B at a non-Factor D cleavage site. In some embodiments, the engineered proteases provided herein target Factor B at a Factor D cleavage site. In some embodiments, the engineered proteases provided herein can target Factor B for cleavage while Factor B is in a complex with C3. In some embodiments, the engineered proteases provided herein can target Factor B for cleavage while Factor B in complex with C3b. In some embodiments, the engineered proteases provided herein can target Factor B for cleavage while Factor B is alone in circulation.

[0066] In some embodiments, cleavage products resulting from cleavage of Factor B by engineered proteases provided herein may be functionally inactive fragments, as discussed above. In some embodiments, the functionally inactive fragments do not have a naturally occurring physiological function. In some embodiments, the functionally inactive fragments may perform a function, but not necessarily the same function as Ba and/or Bb.

[0067] In some embodiments, the engineered proteases provided herein are capable of modulating the activity of the complement system by reducing the amount of Factor B fragments Ba and Bb that are generated, thereby dampening/inhibiting complement activation, limiting an increase complement activation, and/or limiting/reducing the amplification of complement pathways.

[0068] In some embodiments, the engineered proteases provided herein are useful for administration to a subject in need thereof. As used herein, the terms “patient” or “subject” are interchangeably used refer to mammals and include, without limitation, humans and other primates (e.g., chimpanzees, cynomolgus monkeys, and other apes and monkey species), farm animals (e.g., cattle, sheep, pigs, goats and horses), domestic mammals (e.g., dogs and cats), and laboratory animals (e.g., rabbits, rodents such as mice, rats, and guinea pigs). In exemplary embodiments, the subject is a human.

[0069] Table 1 provides the amino acid sequence of a human Factor B, targeted by the engineered proteases of the disclosure.

[0070] Table 1 also provides the amino acid sequences of exemplary chymotrypsin-like serine proteases and protease domains of the S1A family that can be utilized as scaffolds based upon which the engineered proteases of the disclosure are generated, including: MTSP-1, uPA, chymase, and Kallikrein-related peptidase 5 (KLK5). Accordingly, in some embodiments, the engineered proteases provided herein are based on MTSP-1, or the serine protease domain thereof. In some embodiments, the engineered proteases provided herein are based on uPA, or the serine protease domain thereof. In some embodiments, the engineered proteases provided herein are based on KLK5, or the serine protease domain thereof. In some embodiments, the engineered proteases provided herein not based on chymase or a serine protease domain thereof. In some embodiments, the engineered proteases provided herein are not based on MTSP-1 or a serine protease domain thereof. In some embodiments, the engineered proteases provided herein are not based on uPA or a serine protease domain thereof. In some embodiments, the engineered proteases provided herein are not based on KLK5 or a serine protease domain thereof. In some embodiments, the engineered proteases provided herein are not based on chymase or a serine protease domain thereof.

[0071] Table 1 also includes the amino acid sequence of a mature chymotrypsin polypeptide in SEQ ID NO: 19. It is noted that a protease domain of a serine protease can be aligned with that of chymotrypsin such that the amino acid residues of the aligned protease (e.g., MTSP-1, uPA, KLK5, or chymase) correspond to the amino acids of chymotrypsin which are then provided with the numbering of chymotrypsin. This is generally referred to herein as a chymotrypsin numbering, and the numbering, and corresponding positions of aligned proteases can be determined by one of skill in the art. Standard nomenclature useful for chymotrypsin numbering can also be determined by one of skill in the art, such as notations used for additions or deletions or residues. A residue existing in the aligned protease (e.g., MTSP-1, uPA, KLK5, or chymase) which does not exist in chymotrypsin, is provided with a lowercase letter notation. By way of example, using the chymotrypsin numbering key for the uPA protease domain (Table 2), the modification S37dP (chymotrypsin numbering) translates to S184P in conventional amino acid sequence notation. Signal or leader sequences are indicated by underlining, and cleavage site sequences are indicated by bold, in Table 1. In some instances, the disclosure and claims contain reference to conventional amino acid numbering, and/or chymotrypsin based numbering, and is so identified accordingly.

[0072] The chymase of Table 1 is a mast cell chymase, whose sequence can be found at <https://www.uniprot.org/uniprot/P23946>.

Table 1: Human Factor B and Wild Type Serine Protease Sequences

Human Factor B	MGSNLSPQLCLMPFILGLLSGGVTTTPWSLARPGSCSLEGVEI KGGSFRLQEGQALEYVCPSGFYPYPVQTRTCRSTGSWSTLKT QDQKTVRKAECRAIHCPRPHDFENG EYWPRSPYYNV SDEISFH CYDGYTLRGSANRTCQVNGRWSGQTAICDNGAGYCSNPGIPI GTRKVG SQYRLED SVTYHCSRGLTLRGSQRRTCQEGGSWSGT EPSCQDSFMYDTPQEVAEAF LSSLTETIE GVDA EDGHGPGE QQ KRK IVLDPSGSMNIYL VLDGSDSIGASNFTGAKKCLVNLIEKV ASYGVKPRYGLVTYATYPKIWV KVSEAD SSNADWVTKQLNEI NYEDHKLKSGTNTKKALQAVYSMMSWPDDVPPEGWNRTRH VIILMTDGLHNMGGDPITVIDEIRDLLYIGKDRKNPREDYLDVY VFGVGPLVNQVNINALASKKDNEQHVFKVKDMENLEDVFYQ MIDESQSLSLCGMVWEHRKGT DYHKQPWQAKISV IRPSK GHE SCMGAVVSEYFVLTAHCFTVDDKEHSIKVSV GGEKRD LEIE VVL FHPNYNING GKKEAG IPEFYDYDVALIKLKNKLKYGQTIRP ICLPCTEGTTRALRLPPTTTCQQQKEELLPAQDIKALFVSEEEK KLTRKEVYIKNGDKKGSCERDAQYAPGYDKVKDISEVVTPRF LCTGGVSPYADPNTCRGDSGGPLIVHKRSRFIQVGVISWGVVD
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	VCKNQKRQKQVPAHARDFHINLFQVLPWLKEKLQDEDLGFL (SEQ ID NO: 1)
Human Chymase (signal sequence, protease domain)	<u>MRGSHHHHHHGS</u> SDYKDDDDK <u>IIGGTECKPHSRPYMAYLEIVT</u> SNGPSKFCGGFLIRRNFLTA ^{AA} HCAGRSITVTLGAHNITEEEDT WQKLEVIKQFRHPKYNTSTLHHDIMLLKLKEKASLTLAVGTLP FPSQNFVPPGRMCRVAGWGRTGVLKPGSDTLQEVKLRLMDP QACSHFRDFDHNLQLCVGNPRKTKSAFKGDSGGPLLCAGVAQ GIVSYGRSDAKPPAVFTRISHYRPWINQILQAN (SEQ ID NO: 24)
Human Chymase Protease Domain)	IIGGTECKPHSRPYMAYLEIVTSNGPSKFCGGFLIRRNFLTA ^{AA} HCAGRSITVTLGAHNITEEEDTWQKLEVIKQFRHPKYNTSTLH HDIMLLKLKEKASLTLAVGTLPFPSQNFVPPGRMCRVAGWG RTGVLKPGSDTLQEVKLRLMDPQACSHFRDFDHNLQLCVGNP RKTKSAFKGDSGGPLLCAGVAQGIVSYGRSDAKPPAVFTRISH YRPWINQILQAN (SEQ ID NO: 6)
Human MTSP-1 full length	MGSDRARKGGGGPKDFGAGLKYN ^{SR} HEKVNGLEEGVEFLPV NNVKKVEKHGPGRWV ^V LA ^{AV} LIGLLL ^V LLGIGFLV ^{WH} LQYR DVRVQKVFN ^{GY} MRITNENF ^{VD} AYENS ^{NS} TEFV ^{SL} ASKV ^{KD} AL KLLYSGVPFLGPYHKESAV ^{TAF} SEG ^{SV} IAY ^{YW} SEFSIPQHL ^{VEE} AERVMAEERV ^{ML} PPRARS ^{LS} SFV ^{VT} SV ^{VA} FPTDSK ^{TV} QRTQ DN ^{SC} SFGLHARGVELMR ^{FT} TPGF ^{PD} SPYPAHARCQ ^{WA} LRGDA DSVLSL ^{TFR} SFDL ^{AS} CDERGSD ^{LV} TVYNTLSPMEPHALV ^{QL} CG TYP ^{PS} YNLTFHSSQNVLLITLITN ^{TERR} HPGF ^{EAT} FFQLPRMSSC GGRLRKAQGT ^{FN} SPYYPGHYPNIDCTW ^{NIE} VPNNQHVK ^{VR} F KFFYLLEPGVPAG ^{TC} PKDYVEINGEKYCGERSQ ^{FV} VT ^{SN} SNKI TVRFHSDQSY ^{TD} TGFLAEYLSYDSSDPCPGQ ^{FT} CRTGRCIR ^{KEL} RCDGWADCTDHSDELNCSCDAGHQ ^{FT} CKNKFCKPLFW ^{VC} DS VNDCGDN ^{SDE} QGCSCPAQ ^{TF} RCSNGKCLSKSQQCNGKDDCG DGSDEASCPKVN ^{VT} CTKHTYRCLNGLCLSKGNPECDGKEDC SDGSDEKDCDCGLRSF ^{TR} QARV ^{VG} GTDADEGEWPWQV ^{SL} HA LGQGHICGASLISP ^{NWL} VSA ^{AH} CYIDDRGF ^{RY} SDPTQW ^{TAF} LG LHDQSQRSAPGVQERRLKRIISH ^{PF} FNDFTFDYDIALLELEK ^{PA} EYSSMVRPICLPDASHVFPAGKAIW ^{VT} GWGHTQYGGTGALIL QKGEIRVINQ ^{TT} CENLLPQQITPRMMC ^{VG} FLSGGV ^{DSC} QGD ^{SG} GPLSSVEADGRIFQAGV ^{VS} WGDGCAQRNKP ^{GV} YTRLPLFRD ^W IKENTGV (SEQ ID NO: 18)
Human MTSP-1 (signal sequence, protease domain)	<u>MRGSHHHHHHGS</u> DEKDSDSLRSF ^{TR} QARV ^{VG} GTDADEGEW PWQVSLHALGQGHICGASLISP ^{NWL} VSA ^{AH} CYIDDRGF ^{RY} SDP TQW ^{TAF} LG ^{LHD} QSQRSAPGVQERRLKRIISH ^{PF} FNDFTFDYDIA LLELEKPAEYSSMVRPICLPDASHVFPAGKAIW ^{VT} GWGHTQY GGTGALILQKGEIRVINQ ^{TT} CENLLPQQITPRMMC ^{VG} FLSGGV DSCQGD ^{SG} GPLSSVEADGRIFQAGV ^{VS} WGDGCAQRNKP ^{GV} Y RLPLFRDWIKENTGV (SEQ ID NO: 25)
Human MTSP-1	VVGGTDADEGEWPWQVSLHALGQGHICGASLISP ^{NWL} VSA ^{AH} CYIDDRGF ^{RY} SDPTQW ^{TAF} LG ^{LHD} QSQRSAPGVQERRLKRII

Protease Domain	SHPFFNDFTFDYDIALLELEKPAEYSSMVRPICLPDASHVFPAG KAIWVTGWGHTQYGGTGALILQKGEIRVINQTTCENLLPQQIT PRMMC VGFLSGGVDSCQGD SGGLSSVEADGRIFQAGV VSWG DGCAQRNKPGVYTR LPLFRDWIKENTGV (SEQ ID NO: 7)
Human uPA full length	SNELHQVPSNCDCLNGGTCVSNKYFSNIHWCNCPKKFGGQHC EIDKSKTCYEGNGHFYRGKASTDTMGRPCLPWNSATVLQQTY HAHRSDALQLGLGKHNYCRNPDNRRRPWCYVQVGLKPLVQE CMVHDCADGKKPSSPPEELKFQCGQKTLRPRFKIIGGEFTTIEN QPWF AAIYRRHRGGSVTYVCGGSLISPCWVISATHCFIDYPKK EDYIVYLGRSRLNSNTQGEMKFEVENLILHKDYSADTLAHHN DIALLKIRSKEGRCAQPSRTIQ TICLPSMYNDPQFGTSCEITGFG KENSTDYLYPEQLKMTVVKLISHRECQQPHYYGSEVTTKMLC AADPQWKTDSCQGD SGGLVCSLQGRMTLTGIVSWGRGCAL KDKPGVYTRVSHFLPWIRSHSHTKEENGLAL (SEQ ID NO: 8)
Human uPA (signal sequence, protease domain)	MYRMQLLSICIALSLALVTNSIIGGEFTTIENQPWF AAIYRRHRG GSVTYVCGGSLISPCWVISATHCFIDYPKKEDYIVYLGRSRLNS NTQGEMKFEVENLILHKDYSADTLAHHNDIALLKIRSKEGRCA QPSRTIQ TICLPSMYNDPQFGTSCEITGFGKENSTDYLYPEQLK MTVVKLISHRECQQPHYYGSEVTTKMLCAADPQWKTDSCQG DSGGLVCSLQGRMTLTGIVSWGRGCALKDKPGVYTRVSHFL PWIRSHSHTKEENGLAL (SEQ ID NO: 26)
Human uPA Protease Domain	IIGGEFTTIENQPWF AAIYRRHRGGSVTYVCGGSLISPCWVISAT HCFIDYPKKEDYIVYLGRSRLNSNTQGEMKFEVENLILHKDYS ADTLAHHNDIALLKIRSKEGRCAQPSRTIQ TICLPSMYNDPQFG TSCEITGFGKENSTDYLYPEQLKMTVVKLISHRECQQPHYYG SEVTTKMLCAADPQWKTDSCQGD SGGLVCSLQGRMTLTGIVS WGRGCALKDKPGVYTRVSHFLPWIRSHSHTKEENGLA (SEQ ID NO: 22)
Human Kallikrein 5 (KLK5) (signal sequence (underlined), propeptide, and protease domain)	MATARPPWMWVLCALITALLGVTEHVLANNDVSCDHPSNT VPSGSNQDLGAGAGEDARSDDSSSRIINGSDCDMHTQPWQA ALLLRPNQLYCGAVLVHPQWLLTAAHCRKKVFRVRLGHYSL PVYESGQQMFQGVKSIPHPGYSHPGHSNDLMLIKLNRRIRPTK DVRPINVSSHCP SAGTKCLVSGWGTTKSPQVHFPKVLQCLNIS VLSQKRCEDAYPRQIDDTMFCAGDKAGR DSCQGD SGGPVVC NGSLQGLVSWGDYPCARP NRPGVYTNLCKFTKWIQETIQANS (SEQ ID NO: 27)
Human Kallikrein 5 (KLK5) (propeptide, and protease domain)	VTEHVLANNDVSCDHPSNTVPSGSNQDLGAGAGEDARSDDSS SRIINGSDCDMHTQPWQA ALLLRPNQLYCGAVLVHPQWLLTAAHCRKKVFRVRLGHYSL PVYESGQQMFQGVKSIPHPGYSHPGHSNDLMLIKLNRRIRPTK DVRPINVSSHCP SAGTKCLVSGWGTTKSPQVHFPKVLQCLNIS VLSQKRCEDAYPRQIDDTMFCAGDKAGR DSCQGD SGGPVVC

	NGSLQGLVSWGDYPCARPNRPGVYTNLCKFTKWIQETIQANS (SEQ ID NO: 28)
Human Kallikrein 5 Protease Domain	IINGSDCDMHTQPWQA ALLLRPNQLYCGAVLVHPQWLLTAAHCRKKVFRVRLGHYSLS PVYESGQQMFQGVKSIPHPGYSHPGHSNDLMLIKLNRRIRPTK DVRPINVSSHCPASAGTKCLVSGWGTTKSPQVHFPKVLQCLNIS VLSQKRCEDAYPRQIDDTMFCAGDKAGRDSQGDSSGGPVVC NGSLQGLVSWGDYPCARPNRPGVYTNLCKFTKWIQETIQANS (SEQ ID NO: 23)
Chymotrypsin	MAFLWLLSCWALLGTTFGCGVPAIHPVLSGLSRIVNGEDAVPG SWPWQVSLQDKTGFHFCCGSLISEDWVVTAAHCGVRTSDVV VAGEFDQGSDEENIQVLKIAKVFKNPFSILTVNNDITLLKLAT PARFSQTVSAVCLPSADDDFPAGTLCATTGWGKTKYNANKTP DKLQQAALPLLSNAECKKSWGRRITDVMICAGASGVSSCMGD SGGPLVCQKDGAWTLVGIVSWGSDTCSTSSPGVYARVTKLIP WVQKILAAAN (SEQ ID NO: 19)

[0073] FIG. 1A depicts a schematic diagram of a naturally occurring Factor B, showing various cleavage site sequences, including the site at which Factor B is cleaved by Factor D (FD cleavage site) into Ba and Bb. Ba is made up of three complement control protein (CCP) domains, and a linker. Bb is made up of a von Willebrand Factor Type A (VWA) domain, and a serine protease (SP) domain. FIGS. 1B-1C depict two views of the protein structure of Factor B showing various cleavage sites.

[0074] In some embodiments, the disclosure provides engineered proteases that cleave Factor B, which can be at a cleavage site that is not targeted by Factor D, i.e., not at the FD cleavage site. In other embodiments, the disclosure provides engineered proteases that cleave Factor B at a cleavage site that is targeted by Factor D. As contemplated herein, a FD cleavage site is a site that is the amino acid sequence of QQKR/KIV (SEQ ID NO: 9). In other embodiments, the disclosure also provides for engineered proteases that can cleave Factor B at a cleavage site that is targeted by Factor D (SEQ ID NO: 9), before Factor B forms a complex with C3b – without being bound by any theory or mechanism, it is expected that such cleavage can result in fragments that do not increase complement activity.

[0075] Various exemplary cleavage sites of Factor B that may be targeted by the engineered proteases of the disclosure are indicated on the schematic diagram of FIG. 1, and include, but are

not limited to: QQKR/KIV (SEQ ID NO: 9), WEHR/KGT (SEQ ID NO: 10), KNQKR/QKQ (SEQ ID NO: 11), DVFY/QMI (SEQ ID NO: 12), EGVD/AE (SEQ ID NO: 13), DHKL/KSG (SEQ ID NO: 14), TPW/SLA (SEQ ID NO: 15), KVSEAD (SEQ ID NO: 20), IRPSKG (SEQ ID NO: 4), GGEKRD (SEQ ID NO: 5), GKKEAG (SEQ ID NO: 3), and DHKL/KSG (SEQ ID NO: 21). In some exemplary cleavage sites, a slash is used to represent the site of cleavage. However, the cleavage at these sites is not limited to such.

[0076] FIG. 1D depicts a Coomassie gel showing examples of Factor B cleavage by chymase-based engineered proteases. Two lots of each engineered protease set were used, and each lot showed the ability of the engineered proteases to cleave Factor B. FIG. 1E depicts a graph showing examples of Factor B cleavage by two chymase-based engineered proteases having low EC₅₀. These results are discussed in further detail in Example 1 below.

[0077] The amino acid sequence of a wild type human Factor B is presented in Table 1 below, shown by SEQ ID NO: 1. As depicted in Table 1, in some embodiments, a site comprising the amino acid sequence KVSEAD (SEQ ID NO: 2) may be targeted as a cleavage site by an engineered protease of the disclosure. In some embodiments an engineered protease that can target the sequence of SEQ ID NO: 2 for cleavage is based on chymase. In some embodiments, a site comprising the amino acid sequence GKKEAG (SEQ ID NO: 3) may be targeted as a cleavage site by an engineered protease of the disclosure. In some embodiments an engineered protease that can target the sequence of SEQ ID NO: 3 for cleavage is based on MTSP-1 or uPA. In some embodiments, a site comprising the amino acid sequences IRPSKG (SEQ ID NO: 4) and/or GGEKRD (SEQ ID NO: 5) may be targeted as a cleavage site by an engineered protease of the disclosure, in some embodiments such an engineered protease is built on a MTSP-1-based scaffold.

[0078] FIGS. 2A-2B depict schematic diagrams of a pro-chymase and a mature chymase, respectively. The pro-chymase comprises the chymase domain and an activation peptide, while the mature chymase is produced when the signal peptide and the activation peptide are cleaved at the cleavage site indicated in FIG. 2A. The mature chymase as depicted in FIG. 2B can be utilized as a scaffold for generation of engineered proteases of the disclosure. The amino acid sequence of a wild type mature chymase protease domain is presented in Table 1, shown by SEQ ID NO: 6.

[0079] FIGS. 3A-3B depict schematic diagrams of the extracellular portion of Membrane Type Serine Protease 1 (MTSP-1) and the serine protease domain of MTSP-1, respectively. The MTSP-1 serine protease domain, as depicted in FIG. 3B, can be utilized as a scaffold for generation of engineered proteases of the disclosure. The amino acid sequence of the protease domain of a naturally occurring MTSP-1 is presented in SEQ ID NO: 7 in Table 1.

[0080] FIGS. 3C-3D depict schematic diagrams of urokinase-type plasminogen activator (uPA or u-PA). FIG. 3C depicts a schematic diagram of the uPA zymogen, and FIG. 3D depicts a schematic diagram of a mature two chain uPA. The mature uPA polypeptide is generated by proteolytic cleavage. The uPA serine protease domain, as depicted in FIG. 3D, can be utilized as a scaffold for generation of engineered proteases of the disclosure. In wild-type uPA the protease domain is connected to a "A" chain by a disulfide bridge (as depicted in FIG. 3D). In the engineered uPA-based proteases provided herein, comprising a uPA serine protease domain there can be a C to S substitution (C122S as presented by chymotrypsin numbering of SEQ ID NO: 22) to reduce aggregation.

[0081] In some embodiments, provided herein are engineered proteases, wherein the serine proteases are specific for Factor B at a site that is not targeted by Factor D. In other embodiments, provided herein are engineered proteases, wherein the serine proteases are specific for Factor B at a site that is targeted by Factor D. In some embodiments, cleavage of Factor B by the engineered proteases provided herein at the site targeted by Factor D, or at the site not targeted by Factor D, results in a reduction of complement activation. In some embodiments, cleavage at the site generates at least two functionally inactive fragments. In some embodiments, cleavage of Factor B by the engineered proteases provided herein at the site targeted by Factor D, or at the site not targeted by Factor D, results generates one or more functionally inactive fragments. In some embodiments, cleavage of Factor B by the engineered proteases provided herein at the site targeted by Factor D, or at the site not targeted by Factor D, results in a reduction of a function of Factor B. In some embodiments, cleavage of Factor B by the engineered proteases provided herein at the site targeted by Factor D, or at the site not targeted by Factor D, results in a reduction of Factor B cleavage products Ba and Bb.

[0082] In some embodiments, the Factor B targeted by the engineered proteases of the disclosure can be of any species. In some embodiments, the Factor B is a primate Factor B. In some embodiments, the Factor B is human Factor B. In some embodiments, the human Factor B

comprises the amino acid sequence as set forth in SEQ ID NO: 1. In some embodiments, the primate Factor B is a non-human primate Factor B. In some embodiments, the non-human primate is cynomolgus monkey. In some embodiments, the Factor B is a rodent Factor B, e.g., Factor B of a rat, or mouse.

[0083] In some embodiments, the engineered proteases provided herein are specific for Factor B at a site that is not targeted by Factor D, wherein the site targeted by Factor D comprises the amino acid sequence QQKR/KIV (SEQ ID NO: 9). In some embodiments, the site on Factor B that is not targeted by Factor D comprises a sequence selected from the group consisting of WEHR/KGT (SEQ ID NO: 10), KNQKR/QKQ (SEQ ID NO: 11), DVFY/QMI (SEQ ID NO: 12), EGVD/AE (SEQ ID NO: 13), DHKL/KSG (SEQ ID NO: 14), TPW/SLA (SEQ ID NO: 15), KVSEAD (SEQ ID NO: 20), IRPSKG (SEQ ID NO: 4), GGEKRD (SEQ ID NO: 5), GKKEAG (SEQ ID NO: 3), DHKL/KSG (SEQ ID NO: 21), and WEHR/KGT (SEQ ID NO: 10).

[0084] In other embodiments, the engineered proteases provided herein are specific for Factor B at a site targeted by Factor D, wherein the site targeted by Factor D comprises the amino acid sequence QQKR/KIV (SEQ ID NO: 9).

[0085] In some embodiments, the engineered proteases provided herein are based on a chymotrypsin-like serine protease of the S1A family including, but not limited to, membrane type serine protease 1 (MTSP-1), urokinase-type plasminogen activator (uPA), KLK5, and chymase. The engineered proteases of the disclosure comprise modified protease domains based on a scaffold of a serine protease, such as MTSP-1, uPA, KLK5, or chymase.

uPA-Based Engineered Proteases

[0086] In some embodiments, the engineered proteases are based on uPA, e.g. based on a uPA serine protease domain. In some embodiments, such engineered proteases are specific for Factor B at a site that is not targeted by Factor D, for example, wherein the cleavage site comprises a sequence selected from: WEHR/KGT (SEQ ID NO: 10) and KNQKR/QKQ (SEQ ID NO: 11).

[0087] In some embodiments, the uPA-based engineered proteases comprise one or more modifications with respect to a uPA comprising the amino acid sequence as set forth in SEQ ID NO: 8.

[0088] In some embodiments, the uPA-based engineered proteases comprise one or more modifications with respect to a uPA protease domain comprising the amino acid sequence as set forth in SEQ ID NO: 22.

[0089] The modifications to uPA or the uPA protease domain can be referred to by numbering the residues of the uPA protease domain by chymotrypsin numbering. Presented in Table 2 are the corresponding chymotrypsin numbers of the uPA protease domain of SEQ ID NO: 22 (equivalent to the amino acid positions 159-411 of uPA, as set forth in SEQ ID NO: 8).

[0090] Table 2 provides four rows for each amino acid. The first row lists the conventional amino acid sequence numbering of SEQ ID NO: 22, the uPA protease domain. The second row lists the conventional amino acid sequence numbering of residues 159-411 of SEQ ID NO: 8, the uPA protease domain. The third row provides the amino acid single letter abbreviation. The fourth row provides the corresponding chymotrypsin numbering below each amino acid single letter abbreviation. A residue that exists in a protease domain that does not exist in chymotrypsin is represented by a letter at the end of the notation. For example, residues in chymotrypsin that are part of a loop with amino acid 60 based on chymotrypsin numbering which are inserted into an engineered uPA are referred to as D60a, Y60b, P60c.

[0091] Table 2 provides the chymotrypsin numbering schema and its corresponding conventional numbering schema for the uPA protease domain. In subsequent tables, and throughout the disclosure, the modifications to the uPA protease domain are referred to either with chymotrypsin numbering, or with conventional amino acid numbering. If a particular modification is provided only with a chymotrypsin numbering notation, the skilled artisan will understand how to refer to Table 2 and perform the necessary conversion to understand the modification in conventional amino acid terms, and vice versa.

Table 2: Chymotrypsin Numbering of uPA the Protease Domain

Conv No. (SEQ ID 22)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Conv No. (SEQ ID 8)	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173
Amino Acid	I	I	G	G	E	F	T	T	I	E	N	Q	P	W	F
Chymo. No.	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30

Conv No. (SEQ ID 22)	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Conv No. (SEQ ID 8)	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188
Amino Acid	A	A	I	Y	R	R	H	R	G	G	S	V	T	Y	V
Chymo. No.	31	32	33	34	35	36	37	37A	37b	37c	37d	38	39	40	41
Conv No. (SEQ ID 22)	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
Conv No. (SEQ ID 8)	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203
Amino Acid	C	G	G	S	L	I	S	P	C	W	V	I	S	A	T
Chymo. No.	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56
Conv No. (SEQ ID 22)	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
Conv No. (SEQ ID 8)	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218
Amino Acid	H	C	F	I	D	Y	P	K	K	E	D	Y	I	V	Y
Chymo. No.	57	58	59	60	60a	60b	60c	61	62	62a	63	64	65	66	67
Conv No. (SEQ ID 22)	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75
Conv No. (SEQ ID 8)	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233
Amino Acid	L	G	R	S	R	L	N	S	N	T	Q	G	E	M	K
Chymo. No.	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82
Conv No. (SEQ ID 22)	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
Conv No. (SEQ ID 8)	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248
Amino Acid	F	E	V	E	N	L	I	L	H	K	D	Y	S	A	D
Chymo. No.	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97
Conv No. (SEQ ID 22)	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105
Conv No. (SEQ ID 8)	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263
Amino Acid	T	L	A	H	H	N	D	I	A	L	L	K	I	R	S
Chymo. No.	97a	97b	98	99	100	101	102	103	104	105	106	107	108	109	110
Conv No. (SEQ ID 22)	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120
Conv No. (SEQ ID 8)	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278
Amino Acid	K	E	G	R	C	A	Q	P	S	R	T	I	Q	T	I

Chymo. No.	110a	110b	110c	110d	111	112	113	114	115	116	117	118	119	120	121
Conv No. (SEQ ID 22)	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135
Conv No. (SEQ ID 8)	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293
Amino Acid	C	L	P	S	M	Y	N	D	P	Q	F	G	T	S	C
Chymo. No.	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136
Conv No. (SEQ ID 22)	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150
Conv No. (SEQ ID 8)	294	295	296	297	298	299	300	301	302	303	304	305	306	307	308
Amino Acid	E	I	T	G	F	G	K	E	N	S	T	D	Y	L	Y
Chymo. No.	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151
Conv No. (SEQ ID 22)	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165
Conv No. (SEQ ID 8)	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323
Amino Acid	P	E	Q	L	K	M	T	V	V	K	L	I	S	H	R
Chymo. No.	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166
Conv No. (SEQ ID 22)	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180
Conv No. (SEQ ID 8)	324	325	326	327	328	329	330	331	332	333	334	335	336	337	338
Amino Acid	E	C	Q	Q	P	H	Y	Y	G	S	E	V	T	T	K
Chymo. No.	167	168	169	170	170a	170b	171	172	173	174	175	176	177	178	179
Conv No. (SEQ ID 22)	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195
Conv No. (SEQ ID 8)	339	340	341	342	343	344	345	346	347	348	349	350	351	352	353
Amino Acid	M	L	C	A	A	D	P	Q	W	K	T	D	S	C	Q
Chymo. No.	180	181	182	183	184	185	185a	185b	186	187	188	189	190	191	192
Conv No. (SEQ ID 22)	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210
Conv No. (SEQ ID 8)	354	355	356	357	358	359	360	361	362	363	364	365	366	367	368
Amino Acid	G	D	S	G	G	P	L	V	C	S	L	Q	G	R	M
Chymo. No.	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207
Conv No. (SEQ ID 22)	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225

Conv No. (SEQ ID 8)	369	370	371	372	373	374	375	376	377	378	379	380	381	382	383
Amino Acid	T	L	T	G	I	V	S	W	G	R	G	C	A	L	K
Chymo. No.	208	209	210	211	212	213	214	215	216	217	218	220	221	222	223
Conv No. (SEQ ID 22)	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240
Conv No. (SEQ ID 8)	384	385	386	387	388	389	390	391	392	393	394	395	396	397	398
Amino Acid	D	K	P	G	V	Y	T	R	V	S	H	F	L	P	W
Chymo. No.	223a	224	225	226	227	228	229	230	231	232	233	234	235	236	237
Conv No. (SEQ ID 22)	241	242	243	244	245	246	247	248	249	250	251	252	253		
Conv No. (SEQ ID 8)	399	400	401	402	403	404	405	406	407	408	409	410	411		
Amino Acid	I	R	S	H	T	K	E	E	N	G	L	A	L		
Chymo. No.	238	239	240	241	242	243	244	245	246	247	248	249	250		

[0092] The uPA-based engineered proteases of the disclosure comprise at least one modification of the serine protease domain of uPA. As noted above, the modifications can be any one or more of: a deletion of one or more amino acid residues, a deletion of one or more domains, a substitution of one or more amino acid residues, an insertion of one or more amino acid residues, an insertion of one or more domains, and a substitution of one or more domains. Table 3A provides exemplary modifications to the serine protease domain of uPA. By way of example, Table 3A provides three columns – the first column provides the modification using chymotrypsin numbering; the second column provides conventional amino acid sequence numbering, with respect to SEQ ID NO: 8; and the third column provides amino acid sequence numbering, with respect to SEQ ID NO: 22.

[0093] An engineered protease can be generated by the use of any one or more of the exemplary modifications provided in Table 3A. Accordingly, the uPA-based engineered protease of the disclosure may comprise any one or more of the modifications provided in Table 3A.

[0094] In some embodiments, the modification is at any one or more positions corresponding to positions G18, R36, S37, V38, Y40, D60, A96, L97, A98, H99, C122, Y151, V159, A184, Q192, R217, K224, using chymotrypsin numbering. By way of example, a modification to G18E is a substitution of E at the position corresponding to position 18 of the uPA serine protease

domain, using chymotrypsin numbering. By way of example the modification D97delinsEG denotes a deletion of a D at residue 97, and an insertion of EG in its places, using chymotrypsin numbering. By way of example the modification L97b_H99del denotes the deletion of the residues from L97b to H99, using chymotrypsin numbering.

Table 3A: Exemplary Modifications to the Serine Protease Domain of uPA

Chymotrypsin No.	Conventional No. (SEQ ID NO. 8)	Conventional No. (SEQ ID NO. 22)
G18E	G161E	G3E
R36H	R179H	R21H
R36S	R179S	R21S
S37dP	S184P	S26P
V38D	V185D	V27D
Y40H	Y187H	Y29H
Y40N	Y187N	Y29N
D60aR	D208R	D50R
D60aY	D208Y	D50Y
D60aA	D208A	D50A
D60aK	D208K	D50K
D60aH	D208H	D50H
L73R	L224R	L66R
A96del	A247del	A89del
A96_H99del	A247_H252del	A89_H94del
A96_H99delinsPGVE	A247_H252delinsPGVE	A89_H94delinsPGVE
A96D	A247D	A89D
A96E	A247E	A89E
A96N	A247N	A89N
A96G	A247G	A89G
L97bM	L250M	L92M
L97bH	L250H	L92H
L97bY	L250Y	L92Y
L97bI	L250I	L92I
T97a_L97bdel	T249_L250del	T91_L92del
T97a_H99del	T249_H252del	T91_H94del
D97A	D248A	D90A
D97E	D248E	D90E
D97G	D248G	D90G

Chymotrypsin No.	Conventional No. (SEQ ID NO. 8)	Conventional No. (SEQ ID NO. 22)
D97L	D248L	D90L
D97R	D248R	D90R
D97T	D248T	D90T
D97W	D248W	D90W
L97bV	L250V	L92V
L97b_H99del	L250_H252del	L92_H94del
L97bV	L250V	L92V
L97bG	L250G	L92G
D97delinsEG	D249delinsEG	D90delinsEG
T97aS	T249S	T91S
A98_H99del	A251_H252del	A93_H94del
A98G	A251G	A93G
A98N	A251N	A93N
A98T	A251T	A93T
H99A	H252A	H94A
H99E	H252E	H94E
H99K	H252K	H94K
H99L	H252L	H94L
H99M	H252M	H94M
H99N	H252N	H94N
H99P	H252P	H94P
H99Q	H252Q	H94Q
H99R	H252R	H94R
H99S	H252S	H94S
H99T	H252T	H94T
H99V	H252V	H94V
H99W	H252W	H94W
H99Y	H252Y	H94Y
C122S	C279S	C121S
Y151N	Y308	Y150N
V159A	V316A	V158A
Y172L	Y331L	Y173L
A184T	A343T	A185T
Q192A	Q353A	Q195A
Q192C	Q353C	Q195C
Q192D	Q353D	Q195D

Chymotrypsin No.	Conventional No. (SEQ ID NO. 8)	Conventional No. (SEQ ID NO. 22)
Q192E	Q353E	Q195E
Q192F	Q353F	Q195F
Q192G	Q353G	Q195G
Q192H	Q353H	Q195H
Q192I	Q353I	Q195I
Q192K	Q353K	Q195K
Q192L	Q353L	Q195L
Q192M	Q353M	Q195M
Q192N	Q353N	Q195N
Q192P	Q353P	Q195P
Q192R	Q353R	Q195R
Q192S	Q353S	Q195S
Q192T	Q353T	Q195T
Q192W	Q353W	Q195W
Q192V	Q353V	Q195V
Q192Y	Q353Y	Q195Y
R217C	R378C	R220C
R217D	R378D	R220D
R217H	R378H	R220H
R217L	R378L	R220L
R217V	R378V	R220V
R217Y	R378Y	R220Y
K224N	K385N	K227N
K224V	K385V	K227V
K224Y	K385Y	K227Y
K224T	K385T	K227T
K224R	K385R	K227R
K224A	K385A	K227A
A98_H99delinsLK	A251_H252delinsLK	A93_H94delinsLK
A98_H99delinsRS	A251_H252delinsRS	A93_H94delinsRS
H99delinsSPG	H252delinsSPG	H94delinsSPG

[0095] Provided in Table 3B are exemplary modifications (referred to herein as mutation strings) of the disclosure. Accordingly, provided here are uPA-based engineered proteases comprising one or more modifications provided in Table 3A. Such exemplary engineered

proteases may be capable of cleaving Factor B, or display other cleavage activity. The second column provides the exemplary modification combinations using conventional numbering, with respect to SEQ ID NO: 22.

Table 3B: Exemplary uPA-Based Engineered Proteases

Chymotrypsin No.	Conventional No. (SEQ ID NO. 22)
C122S	C121S
H99L/C122S	H94L/C121S
H99P/C122S	H94P/C121S
G18E/R36S/V38D/C122S/V159A	G3E/R21S/V27D/C121S/V158A
H99D/C122S	H94D/C121S
H99N/C122S	H94N/C121S
H99C/C122S	H94C/C121S
C122S/R217V	C121S/R220V
R36H/S37dP/V38D/C122S/A184T	R21H/S26P/V27D/C121S/A185T
V38D/A96E/D97G/C122S/Y172L/A98G/H99M/T97a_L97bdel	V27D/A89E/D90G/C121S/Y173L/A93G/H94M/T91_L92del
V38D/C122S/T97a_L97bdel	V27D/C121S/T91_L92del
V38D/A96G/D97E/A98G/H99V/C122S/T97a_L97bdel	V27D/A89G/D90E/A93G/H94V/C121S/T91_L92del
C122S/V38D/A96N/D97A/A98G/H99V/T97a_L97bdel	C121S/V27D/A89N/D90A/A93G/H94V/T91_L92del
V38D/C122S/D97L/A98G/H99V/T97a_L97bdel	V27D/C121S/D90L/A93G/H94V/T91_L92del
V38D/C122S/A96E/D97R/A98G/H99V/T97a_L97bdel	V27D/C121S/A89E/D90R/A93G/H94V/T91_L92del
V38D/C122S/A96E/D97H/A98G/H99L/T97a_L97bdel	V27D/C121S/A89E/D90H/A93G/H94L/T91_L92del
V38D/A96E/D97E/C122S/A98G/H99A/T97a_L97bdel	V27D/A89E/D90E/C121S/A93G/H94A/T91_L92del
V38D/C122S/A96D/D97G/A98G/H99A/T97a_L97bdel	V27D/C121S/A89D/D90G/A93F/H94A/T91_L92del
V38D/D97A/C122S/A98G/H99L/T97a_L97bdel	V27D/D90A/C121S/A93G/H94L/T91_L92del
V38D/C122S/A96D/D97W/A98N/H99L/T97a_L97bdel	V27D/C121S/A89D/D90W/A93N/H94L/T91_L92del
V38D/C122S/A96D/D97T/A98G/H99M/T97a_L97bdel	V27D/C121S/A89G/H94M/T91_L92del

Chymotrypsin No.	Conventional No. (SEQ ID NO. 22)
V38D/D97E/L97bV/A98G/H99V/C122S/A96_H99del	V27D/D90E/L92V/A93G/H94V/C121S/A89_H94del
V38D/A96G/D97A/H99E/C122S/L97b_H99del	V27D/A89G/D90A/H94E/C121S/L92_H94del
V38D/C122S/A96_H99delinsPGVE	V27D/C121S/A89_H94delinsPGVE
V38D/C122S/L97b_H99del/D97delinsEG	V27D/C121S/L92_H94del/D90delinsEG
V38D/T97aS/L97bV/A98T/H99E/C122S/A96_H99del	V27D/T91S/L92V/A93T/H94E/C121S/A89_H94del
V38D/C122S/A96del/A98_H99del/-nulldelinsLK	V27D/C121S/A89del/A93_H94del/-nulldelinsLK
V38D/A96G/D97E/A98G/H99E/C122S/T97a_H99del	V27D/A89G/D90E/A93G/H94E/C121S/T91_H94del
V38D/C122S/A96del/A98_H99del/-nulldelinsRS	V27D/C121S/A89del/A93_H94del/-nulldelinsRS
V38D/D97E/C122S/-null_H99delinsSPG	V27D/D90E/C121S/-null_H94delinsSPG
Y40H/C122S	Y29H/C121S
V38D/A98G/H99A/C122S/T97a_L97bdel	V27D/A93G/H94A/C121S/T91_L92del
V38D/A98G/H99A/C122S/L97bdel	V27D/A93G/H94A/C121S/L92del
V38D/A98G/H99L/C122S/T97a_L97bdel	V27D/A93G/H94L/C121S/T91_L92del

[0096] In some embodiments, a uPA-based engineered protease of the disclosure comprises at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 95% sequence identity to SEQ ID NO: 8.

[0097] In some embodiments, a uPA-based engineered protease of the disclosure comprises at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 95% sequence identity to SEQ ID NO: 22.

[0098] In some embodiments, a uPA-based engineered protease of the disclosure comprises a protease domain comprising at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 95% sequence identity to SEQ ID NO: 22.

MTSP-1-Based Engineered Proteases

[0099] In some embodiments, the engineered proteases are based on MTSP-1, e.g. based on a modified MTSP-1 serine protease domain. In some embodiments, such engineered proteases are specific for Factor B at a site that is not targeted by Factor D, wherein the cleavage site comprises a sequence selected from: WEHR/KGT (SEQ ID NO: 10) and KNQKR/QKQ (SEQ ID NO: 11).

[0100] In some embodiments, the engineered MTSP-1 proteases comprise one or more modifications with respect to a MTSP-1 comprising the amino acid sequence as set forth in SEQ ID NO: 18.

[0101] In some embodiments, the MTSP-1-based engineered proteases comprise one or more modifications with respect to a MTSP-1 protease domain comprising the amino acid sequence as set forth in SEQ ID NO: 7.

[0102] The modifications to the MTSP-1 or MTSP-1 protease domain can be referred to by numbering the residues of the MTSP-1 protease domain by chymotrypsin numbering. Presented in Table 4 are the corresponding chymotrypsin numbers of the MTSP-1 protease domain of SEQ ID NO: 7 (equivalent to the amino acid positions 615-855 of MTSP-1 as set forth in SEQ ID NO: 18).

[0103] Table 4 provides four rows for each amino acid. The first row lists the conventional amino acid sequence numbering of SEQ ID NO: 7, the MTSP-1 protease domain. The second row lists the conventional amino acid sequence numbering of residues 615-855 of SEQ ID NO: 18, the MTSP-1 protease domain. The third row provides the amino acid single letter abbreviation. The fourth row provides the corresponding chymotrypsin numbering below each amino acid single letter abbreviation. A residue that exists in a protease domain that does not exist in chymotrypsin is represented by a letter at the end of the notation. For example, residues in chymotrypsin that are part of a loop with amino acid 60 based on chymotrypsin numbering which are inserted into an engineered MTSP-1 are referred to as D60a and R60c.

[0104] Table 4 provides the chymotrypsin numbering schema and its corresponding conventional numbering schema for the MTSP-1 protease domain. In subsequent tables, and throughout the disclosure, the modifications to the MTSP-1 protease domain are referred to

either with chymotrypsin numbering, or using conventional amino acid numbering. If a particular modification is provided only with a chymotrypsin numbering notation, the skilled artisan will understand how to refer to Table 4 and perform the necessary conversion to understand the modification in conventional amino acid terms, and vice versa.

Table 4: Chymotrypsin Numbering of the MTSP-1 Protease Domain

Conv No. (SEQ ID 7)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Conv No. (SEQ ID 18)	615	616	617	618	619	620	621	622	623	624	625	626	627	628	629
Amino Acid	V	V	G	G	T	D	A	D	E	G	E	W	P	W	Q
Chymo. No.	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Conv No. (SEQ ID 7)	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Conv No. (SEQ ID 18)	630	631	632	633	634	635	636	637	638	639	640	641	642	643	644
Amino Acid	V	S	L	H	A	L	G	Q	G	H	I	C	G	A	S
Chymo. No.	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
Conv No. (SEQ ID 7)	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
Conv No. (SEQ ID 18)	645	646	647	648	649	650	651	652	653	654	655	656	657	658	659
Amino Acid	L	I	S	P	N	W	L	V	S	A	A	H	C	Y	I
Chymo. No.	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
Conv No. (SEQ ID 7)	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
Conv No. (SEQ ID 18)	660	661	662	663	664	665	666	667	668	669	670	671	672	673	674
Amino Acid	D	D	R	G	F	R	Y	S	D	P	T	Q	W	T	A
Chymo. No.	60a	60b	60c	60d	60e	60f	60g	60h	60i	61	62	63	64	65	66
Conv No. (SEQ ID 7)	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75
Conv No. (SEQ ID 18)	675	676	677	678	679	680	681	682	683	684	685	686	687	688	689
Amino Acid	F	L	G	L	H	D	Q	S	Q	R	S	A	P	G	V
Chymo. No.	67	68	69	70	71	72	73	74	74a	75	76	77	78	79	80
Conv No. (SEQ ID 7)	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90

Conv No. (SEQ ID 18)	690	691	692	693	694	695	696	697	698	699	700	701	702	703	704	
Amino Acid	Q	E	R	R	L	K	R	I	I	S	H	P	F	F	N	
Chymo. No.	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	
Conv No. (SEQ ID 7)	91	92			94	95	96	97	98	99	100	101	102	103	104	105
Conv No. (SEQ ID 18)	705	706			707	708	709	710	711	712	713	714	715	716	717	718
Amino Acid	D	F	T	L	T	F	D	Y	D	I	A	L	L	E	L	E
Chymo. No.	96	97	97a	97b	98	99	100	101	102	103	104	105	106	107	108	109
Conv No. (SEQ ID 7)	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	
Conv No. (SEQ ID 18)	719	720	721	722	723	724	725	726	727	728	729	730	731	732	733	
Amino Acid	K	P	A	E	Y	S	S	M	V	R	P	I	C	L	P	
Chymo. No.	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	
Conv No. (SEQ ID 7)	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	
Conv No. (SEQ ID 18)	734	735	736	737	738	739	740	741	742	743	744	745	746	747	748	
Amino Acid	D	A	S	H	V	F	P	A	G	K	A	I	W	V	T	
Chymo. No.	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	
Conv No. (SEQ ID 7)	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	
Conv No. (SEQ ID 18)	749	750	751	752	753	754	755	756	757		758	759	760	761	762	
Amino Acid	G	W	G	H	T	Q	Y	G	G		T	G	A	L	I	
Chymo. No.	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	
Conv No. (SEQ ID 7)	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	
Conv No. (SEQ ID 18)	763	764	765	766	767	768	769	770	771	772	773	774	775	776	777	
Amino Acid	L	Q	K	G	E	I	R	V	I	N	Q	T	T	C	E	
Chymo. No.	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	
Conv No. (SEQ ID 7)	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	
Conv No. (SEQ ID 18)	778	779	780	781	782	783	784	785	786	787	788	789	790	791	792	
Amino Acid	N	L	L	P	Q	Q	I	T	P	R	M	M	C	V	G	
Chymo. No.	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	

Conv No. (SEQ ID 7)	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195
Conv No. (SEQ ID 18)	793	794	795	796	797	798	799	800	801	802	803	804	805	806	807
Amino Acid	F	L	S	G	G	V	D	S	C	Q	G	D	S	G	G
Chymo. No.	184a	185	186	186a	187	188	189	190	191	192	193	194	195	196	197
Conv No. (SEQ ID 7)	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210
Conv No. (SEQ ID 18)	808	809	810	811	812	813	814	815	816	817	818	819	820	821	822
Amino Acid	P	L	S	S	V	E	A	D	G	R	I	F	Q	A	G
Chymo. No.	198	199	200	201	202	203	204	204a	205	206	207	208	209	210	211
Conv No. (SEQ ID 7)	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225
Conv No. (SEQ ID 18)	823	824	825	826	827	828	829	830	831	832	833	834	835	836	837
Amino Acid	V	V	S	W	G	D	G	C	A	Q	R	N	K	P	G
Chymo. No.	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226
Conv No. (SEQ ID 7)	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240
Conv No. (SEQ ID 18)	838	839	840	841	842	843	844	845	846	847	848	849	850	851	852
Amino Acid	V	Y	T	R	L	P	L	F	R	D	W	I	K	E	N
Chymo. No.	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241
Conv No. (SEQ ID 7)	241	242	243												
Conv No. (SEQ ID 18)	853	854	855												
Amino Acid	T	G	V												
Chymo. No.	242	243	244												

[0105] The MTSP-1-based engineered proteases of the disclosure comprise at least one modification of the serine protease domain of MTSP-1. As noted above, the modifications can be any one or more of: a deletion of one or more amino acid residues, a deletion of one or more domains, a substitution of one or more amino acid residues, an insertion of one or more amino acid residues, an insertion of one or more domains, and a substitution of one or more domains. Table 5A provides exemplary modifications to the serine protease domain of MTSP-1. By way of example, Table 5A provides three columns – the first column provides the modification using

chymotrypsin numbering; the second column provides conventional amino acid sequence numbering, with respect to SEQ ID NO: 18; and the third column provides amino acid sequence numbering, with respect to SEQ ID NO: 7.

[0106] The modifications to MTSP-1 or the MTSP-1 protease domain can be referred to by numbering the residues of the MTSP-1 protease domain by chymotrypsin numbering. Presented in Table 4 are the corresponding chymotrypsin numbers of the MTSP-1 protease domain of SEQ ID NO: 7 (equivalent to the amino acid positions 615-855 of MTSP-1, as set forth in SEQ ID NO: 18).

[0107] An engineered protease can be generated by the use of any one or more of the exemplary modifications provided in Table 5A. Accordingly, the MTSP-1-based engineered protease of the disclosure may comprise any one or more of the modifications provided in Table 5A.

[0108] In some embodiments, the modification is at any one or more positions corresponding to positions D23, I41, L70, A77, F94, D96, F97, T98, F99, K110, C122, D125, Y146, Q175, V183, Q192, A204, D217, and K224, using chymotrypsin numbering. By way of example, a modification to F99S of MTSP-1 is a substitution modification at the position corresponding to position 99 of the MTSP-1 serine protease domain, using chymotrypsin numbering.

Table 5A: Exemplary Modifications to the Serine Protease Domain of MTSP-1

Chymotrypsin No.	Conventional No. (SEQ ID NO. 18)	Conventional No. (SEQ ID NO. 7)
D23Y	D622Y	D8Y
I41A	I640A	I26A
I41F	I640F	I26F
I41G	I640G	I26G
I41K	I640K	I26K
I41N	I640N	I26N
I41Q	I640Q	I26Q
I41R	I640R	I26R
I41S	I640S	I26S
L70Q	L678Q	L64
A77T	A686T	A72T
F94Y	F703Y	F89Y

Chymotrypsin No.	Conventional No. (SEQ ID NO. 18)	Conventional No. (SEQ ID NO. 7)
D96A	D705A	D91A
D96P	D705P	D91P
D96S	D705S	D91S
D96T	D705T	D91T
D96insD	D705insD	D91insD
F97A	F706A	F92A
F97D	F706D	F92D
F97E	F706E	F92E
F97G	F706G	F92G
F97K	F706K	F92K
F97L	F706L	F92L
F97M	F706M	F92M
F97R	F706R	F92R
F97S	F706S	F92S
F97T	F706T	F92T
ins 97aA	ins 706aA	ins 93A
ins 97aS	ins 706aS	ins 93S
ins 97aC	ins 706aC	ins 93C
ins 97bG	ins 706bG	ins 93aG
ins 97bL	ins 706bL	ins 93aL
ins 97bN	ins 706bN	ins 93aN
ins 97aV	ins 706aV	ins 93V
ins 97aY	ins 706aY	ins 93Y
ins 97bS	ins 706bS	ins 93aS
ins 97bT	ins 706bT	ins 93aT
T98insA	T707insA	T94insA
T98insAA	T707insAA	T94insAA
T98A	T707A	T94A
T98G	T707G	T94G
T98P	T707P	T94P
T98S	T707S	T94S
T98V	T707V	T94V
F99H	F708H	F95H
F99I	F708I	F95I
F99L	F708L	F95L
F99M	F708M	F95M

Chymotrypsin No.	Conventional No. (SEQ ID NO. 18)	Conventional No. (SEQ ID NO. 7)
F99N	F708N	F95N
F99P	F708P	F95P
F99S	F708S	F95S
F99V	F708V	F95V
F99Y	F708Y	F95Y
K110I	K719I	K106I
C122S	C731S	C118S
D125G	D734G	D121G
Y146D	Y755D	Y142D
Y146E	Y755E	Y142E
R161G	R769G	R157G
L172M	L780M	L168M
Q175H	Q783H	Q171H
Q175L	Q783L	Q171L
Q175N	Q783N	Q171N
Q175P	Q783P	Q171P
Q175R	Q783R	Q171R
Q175S	Q783S	Q171S
Q175T	Q783T	Q171T
Q175W	Q783W	Q171W
V183A	V791A	V179A
Q192M	Q802M	Q190M
Q192R	Q802R	Q190R
Q192T	Q802T	Q190T
Q192V	Q802V	Q190V
G197A	G807A	G195A
A204V	A814V	A202V
D217F	D828F	D216F
D217I	D828I	D216I
D217M	D828M	D216M
D217T	D828T	D216T
D217Y	D828Y	D216Y
D217V	D828V	D216V
D217Y	D828Y	D216Y
K224A	K835A	K223A
K224G	K835G	K223G

Chymotrypsin No.	Conventional No. (SEQ ID NO. 18)	Conventional No. (SEQ ID NO. 7)
K224L	K835L	K223L
K224M	K835M	K223M
K224N	K835N	K223N
K224Q	K835Q	K223Q
K224R	K835R	K223R
K224T	K835T	K223T
K224Y	K835Y	K223Y

[0109] Provided in Table 5B are exemplary modifications (mutation strings) of the disclosure. Accordingly, provided here are MTSP-1-based engineered proteases, comprising one or more mutation strings provided in Table 5B. Such exemplary engineered proteases may be capable of cleaving Factor B, or display other cleavage activity. Residues that are noted in brackets, such as C[17] and C[19], refer to residues that are part of the chain of the protease in zymogen form, which is later cleaved and does not remain in the mature protease.

Table 5B: Exemplary MTSP-1-Based Engineered Proteases

Chymotrypsin No.
C122S
Y146E/K224N
F97E/K224N/F99L/D217I/C122S/C[17]S/C[19]S
C[17]S/C[19]S/F97E/F99L/C122S/Q175L/Q192M/D217I/K224R
C[17]S/C[19]S/F97M/F99L/C122S/Q175W/D217I/K224A
F97E/K224N/F99L/D217I/C122S
F97E/F99L/C122S/Q175L/Q192M/D217I/K224R
F97M/F99L/C122S/Q175W/D217I/K224A
I41G/F97D/F99L/C122S
A77aT/F94Y/C122S/D125G
I41A/F97D/F99L/C122S
I41S/F97D/F99V/C122S/Y146D/C[17]S/C[19]S
I41G/F97D/F99L/C122S/Y146D/C[17]S/C[19]S
I41G/F97E/F99L/C122S/C[17]S/C[19]S
I41N/F97D/F99L/C122S/C[17]S/C[19]S
I41A/F97D/F99V/C122S/Y146D/Q175T/C[17]S/C[19]S
I41G/F97D/F99M/C122S/C[17]S/C[19]S

Chymotrypsin No.
F97L/T98G/F99V/C122S/C[17]S/C[19]S/-null_D96insD
C122S/C[17]S/C[19]S/-null_T98insA
C122S/C[17]S/C[19]S/-null_T98insAA
C[17]S/C[19]S/I41Q/F97T/F99L/C122S/Y146D/Q175W
C[17]S/C[19]S/I41F/F97D/F99I/C122S/Y146D/Q175S/D217T
C[17]S/C[19]S/I41R/F97D/F99N/C122S/Y146D/D217V/K224Q
C[17]S/C[19]S/I41R/F97E/F99N/C122S/Y146D/D217Y/K224M
C[17]S/C[19]S/I41G/F97E/F99L/C122S/R161G/D217Y/K224Q
C[17]S/C[19]S/I41R/F97D/F99H/C122S/Y146D/Q175P/Q192M/D217F/K224L
C[17]S/C[19]S/I41R/F97E/F99S/C122S/Y146D/Q175R/D217I/K224M
C[17]S/C[19]S/I41K/F97D/F99Y/C122S
I41K/F97D/F99H/C122S/C[17]S/C[19]S
F94Y/F99L/C122S/G197A/C[17]S/C[19]S
F99L/C122S/L172M/G197A/C[17]S/C[19]S
D23Y/L70Q/F99L/K110I/C122S/V183A/A204V/C[17]S/C[19]S
F94Y/C122S/C[17]S/C[19]S
I41A/F97D/F99L/C122S
I41N/F97D/F99L/C122S/C[17]S/C[19]S
I41S/F97D/F99V/C122S/Y146D
I41G/F97D/F99L/C122S/Y146D/
I41G/F97E/F99L/C122S
I41N/F97D/F99L/C122S
I41A/F97D/F99V/C122S/Y146D/Q175T
I41G/F97D/F99M/C122S
F97L/T98G/F99V/C122S/-null_D96insD
C122S/-null_T98insA
C122S/-null_T98insAA
I41Q/F97T/F99L/C122S/Y146D/Q175W
I41F/F97D/F99I/C122S/Y146D/Q175S/D217T
I41R/F97D/F99N/C122S/Y146D/D217V/K224Q
I41R/F97E/F99N/C122S/Y146D/D217Y/K224M
I41G/F97E/F99L/C122S/R161G/D217Y/K224Q
I41R/F97D/F99H/C122S/Y146D/Q175P/Q192M/D217F/K224L
I41R/F97E/F99S/C122S/Y146D/Q175R/D217I/K224M
I41K/F97D/F99Y/C122S
I41K/F97D/F99H/C122S

Chymotrypsin No.
F94Y/F99L/C122S/G197A
F99L/C122S/L172M/G197A
D23Y/L70Q/F99L/K110I/C122S/V183A/A204V
F94Y/C122S
I41A/F97D/F99L/C122S
I41N/F97D/F99L/C122S
Y146E/K224N

[0110] In some embodiments, a MTSP-1-based engineered protease of the disclosure comprises at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 95% sequence identity to SEQ ID NO: 18.

[0111] In some embodiments, a MTSP-1-based engineered protease of the disclosure comprises at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 95% sequence identity to SEQ ID NO: 7.

[0112] In some embodiments, a MTSP-1-based engineered protease of the disclosure comprises a protease domain comprising at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 95% sequence identity to SEQ ID NO: 7.

Chymase-Based Engineered Proteases

[0113] In some embodiments, the engineered proteases are based on chymase, e.g. based on a modified chymase serine protease domain. In some embodiments, such engineered proteases are specific for Factor B at a site that is not targeted by Factor D, wherein the cleavage site comprises a sequence selected from: DVFY/QMI (SEQ ID NO: 12), EGVD/AE (SEQ ID NO: 13), DHKL/KSG (SEQ ID NO: 14), and TPW/SLA (SEQ ID NO: 15).

[0114] In some embodiments, the chymase-based engineered proteases comprise one or more modifications with respect to a chymase protease domain comprising the amino acid sequence as set forth in SEQ ID NO: 6.

[0115] The modifications to the chymase or the chymase protease domain can be referred to by numbering the residues of the chymase protease domain by chymotrypsin numbering. Presented in Table 6 are the corresponding chymotrypsin numbers of amino acid positions 1-226 of the chymase protease domain of SEQ ID NO: 6.

[0116] Table 6 provides three rows for each amino acid. The first row lists the conventional amino acid sequence numbering of SEQ ID NO: 6, the chymase protease domain. The second row provides the amino acid single letter abbreviation. The third row provides the corresponding chymotrypsin numbering of the chymase protease domain below each amino acid single letter abbreviation. A residue that exists in a protease domain that does not exist in chymotrypsin is represented by a letter at the end of the notation. For example, residues in chymotrypsin at amino acid 36 based on chymotrypsin numbering which are inserted into an engineered chymase are referred to as V36a, S36b, and N36c.

[0117] Table 6 provides the chymotrypsin numbering schema and its corresponding conventional numbering schema for the Chymase protease domain. In subsequent tables, and throughout the disclosure, the modifications to the Chymase protease domain are referred to either with chymotrypsin numbering, or using conventional amino acid numbering. If a particular modification is provided only with a chymotrypsin numbering notation, the skilled artisan will understand how to refer to Table 6 and perform the necessary conversion to understand the modification in conventional amino acid terms, and vice versa.

Table 6: Chymotrypsin Numbering of the Chymase Protease Domain

Conv No. (SEQ ID 6)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Amino Acid	I	I	G	G	T	E	C	K	P	H	S	R	P	Y	M
Chymo. No.	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Conv No. (SEQ ID 6)	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Amino Acid	A	Y	L	E	I	V	T	S	N	G	P	S	K	F	C
Chymo. No.	31	32	33	34	35	36	36a	36b	36c	37	38	39	40	41	42

Conv No. (SEQ ID 6)	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
Amino Acid	G	G	F	L	I	R	R	N	F	V	L	T	A	A	H
Chymo. No.	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57
Conv No. (SEQ ID 6)	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
Amino Acid	C	A	G	R	S	I	T	V	T	L	G	A	H	N	I
Chymo. No.	58	59	60	61	63	64	65	66	67	68	69	70	71	72	73
Conv No. (SEQ ID 6)	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75
Amino Acid	T	E	E	E	D	T	W	Q	K	L	E	V	I	K	Q
Chymo. No.	74	75	77	78	79	79a	80	81	82	83	84	85	86	87	88
Conv No. (SEQ ID 6)	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
Amino Acid	F	R	H	P	K	Y	N	T	S	T	L	H	H	D	I
Chymo. No.	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103
Conv No. (SEQ ID 6)	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105
Amino Acid	M	L	L	K	L	K	E	K	A	S	L	T	L	A	V
Chymo. No.	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118
Conv No. (SEQ ID 6)	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120
Amino Acid	G	T	L	P	F	P	S	Q	F	N	F	V	P	P	G
Chymo. No.	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133
Conv No. (SEQ ID 6)	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135
Amino Acid	R	M	C	R	V	A	G	W	G	R	T	G	V	L	K
Chymo. No.	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148
Conv No. (SEQ ID 6)	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150
Amino Acid	P	G	S	D	T	L	Q	E	V	K	L	R	L	M	D
Chymo. No.	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164
Conv No. (SEQ ID 6)	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165
Amino Acid	P	Q	A	C	S	H	F	R	D	F	D	H	N	L	Q
Chymo. No.	165	166	167	168	169	172	173	174	175	176	177	177a	178	179	180

Conv No. (SEQ ID 6)	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180
Amino Acid	L	C	V	G	N	P	R	K	T	K	S	A	F	K	G
Chymo. No.	181	182	183	184	185	185a	185b	186	187	188	189	190	191	192	193
Conv No. (SEQ ID 6)	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195
Amino Acid	D	S	G	G	P	L	L	C	A	G	V	A	Q	G	I
Chymo. No.	194	195	196	197	198	199	200	201	202	207	208	209	210	211	212
Conv No. (SEQ ID 6)	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210
Amino Acid	V	S	Y	G	R	S	D	A	K	P	P	A	V	F	T
Chymo. No.	213	214	215	216	217	218	219	220	221	224	225	226	227	228	229
Conv No. (SEQ ID 6)	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225
Amino Acid	R	I	S	H	Y	R	P	W	I	N	Q	I	L	Q	A
Chymo. No.	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244
Conv No. (SEQ ID 6)	226														
Amino Acid	N														
Chymo. No.	245														

[0118] The chymase-based engineered proteases of the disclosure comprise at least one modification of the serine protease domain of chymase. As noted above, the modifications can be any one or more of: a deletion of one or more amino acid residues, a deletion of one or more domains, a substitution of one or more amino acid residues, an insertion of one or more amino acid residues, an insertion of one or more domains, and a substitution of one or more domains. Table 7A provides exemplary modifications to the serine protease domain of chymase. By way of example, Table 7A provides two columns – the first column provides the modifications using chymotrypsin numbering; the second column provides conventional amino acid sequence numbering, with respect to SEQ ID NO: 6.

[0119] An engineered protease can be generated by the use of any one or more of the exemplary modifications provided in Table 7A. Accordingly, the chymase-based engineered protease of the disclosure may comprise any one or more of the modifications provided in Table 7A.

[0120] In some embodiments, the modification is at any one or more positions corresponding to positions C22, S36, P38, G43, R49, K87, K93, I103, L114, L116, F123, V138, F173, D175, S189, A190, F191, K192, L199, V213, G216, A220, A226, F228, using chymotrypsin numbering. By way of example, a modification to C22S of chymase is a substitution modification at the position corresponding to position 22 of the chymase serine protease domain, using chymotrypsin numbering.

Table 7A: Exemplary Modifications to the Serine Protease Domain of Chymase

Chymotrypsin No.	Conventional No. (SEQ ID NO. 6)
C22S	C7S
G43S	G31S
R49P	R37P
S36bN	S23N
P38A	P26A
P38Q	P26Q
P38S	P26S
K87E	K74E
K93E	K80E
K93M	K80M
L99G	L86G
I103V	I90V
L114P	L101P
F123L	F110L
V138A	V125A
V138I	V125I
V138L	V125L
V138M	V125M
V138S	V125S
L116P	L103P
G145D	G132D
L160V	L146V
F173A	F157A
F173G	F157G
F173H	F157H

Chymotrypsin No.	Conventional No. (SEQ ID NO. 6)
F173L	F157L
F173R	F157R
F173Y	F157Y
S189A	S176A
S189E	S176E
S189G	S176G
S189K	S176K
S189L	S176L
S189M	S176M
S189Q	S176Q
S189R	S176R
S189T	S176T
S189V	S176V
S189Y	S176Y
A190D	A177D
A190E	A177E
A190H	A177H
A190I	A177I
A190L	A177L
A190M	A177M
A190P	A177P
A190S	A177S
A190T	A177T
A190V	A177V
F191A	F178A
F191D	F178D
F191E	F178E
F191G	F178G
F191H	F178H
F191I	F178I
F191L	F178L
F191Q	F178Q
F191R	F178R

Chymotrypsin No.	Conventional No. (SEQ ID NO. 6)
F191S	F178S
F191T	F178T
F191V	F178V
F191W	F178W
F191Y	F178Y
K192A	K179A
K192I	K179I
K192L	K179L
K192M	K179M
K192R	K179R
K192S	K179S
L199V	L186V
V213A	V196A
V213G	V196G
Y215F	Y198F
Y215S	Y198S
Y215L	Y198L
Y215T	Y198T
G216A	G199A
G216I	G199I
G216L	G199L
G216M	G199M
G216N	G199N
G216Q	G199Q
G216S	G199S
G216T	G199T
G216V	G199V
G216Y	G199Y
R217A	R200A
R217G	R200G
R217K	R200K
R217P	R200P
S218D	S201D
S218F	S201F

Chymotrypsin No.	Conventional No. (SEQ ID NO. 6)
S218H	S201H
S218M	S201M
A220D	A203D
A220E	A203E
A220G	A203G
A220H	A203H
A220I	A203I
A220K	A203K
A220L	A203L
A220M	A203M
A220N	A203N
A220Q	A203Q
A220R	A203R
A220T	A203T
A220V	A203V
A220W	A203W
A220Y	A203Y
A226D	A207D
A226E	A207E
A226F	A207F
A226G	A207G
A226I	A207I
A226L	A207L
A226M	A207M
A226N	A207N
A226R	A207R
A226S	A207S
A226T	A207T
A226V	A207V
A226Y	A207Y
F228A	F209A
L99H	L86S
Y215H	Y198H

Chymotrypsin No.	Conventional No. (SEQ ID NO. 6)
R217T	R200T
S218V	S201V
F173I	F157I
Y215K	Y198K
S218I	S201I
F41I	F29I
L99W	L86W
G151L	G137L
F173K	F157K
R217S	R200S
F41R	F29R
G151T	G137T
Y215R	Y198R
R217M	R200M
F41V	F29V
L99Y	L86Y
L99F	L86F
G151R	G137R
G151V	G137V
L99R	L86R
S218L	S201L
K40L	K28L
F41A	F29A
F41G	F29G
K40M	K28L
S218R	S201R
L99Q	L86Q
G151S	G137S
G151K	G137K
F173S	F157S
F41K	F29K
G151A	G137A
F41L	F29L

Chymotrypsin No.	Conventional No. (SEQ ID NO. 6)
K93Q	K80Q
G119D	G106D
H100P	H87P
L116R	L103R
Q180H	Q165H
R235S	R216S
E21G	E6G
R61H	R49H
M135T	M122T
D175N	D159N
A209V	A192V
K111E	K98E
P150S	P136S
S152G	S138G
Q166H	Q152H
D175N	D159N
A220S	A203S
L99N	L86N
G151P	G137P
F41H	F29H
S218E	S201E
V183I	V168I
F228V	F209V
V138T	V125T
A190G	A177G
F228M	F209M
D175S	D159S
D175W	D159W
D175R	D159R
S218T	S201T
D175Y	D159Y
V213Q	V196Q
K40A	K28A

Chymotrypsin No.	Conventional No. (SEQ ID NO. 6)
L99M	L86M
D175H	D159H
D175T	D159T
F173M	F157M
D175T	D159T
D175A	D159A
K40V	K28V
F41T	F29T
D175K	D159K
D175Q	D159Q
F41Q	F29Q
D175W	D159W
F41H	F29H
D175S	D159S
S218N	S201N
K40Q	K28Q
K40I	K28I
D175F	D159F
L99S	L86S
G151H	G137H

[0121] Provided in Table 7B are exemplary modifications (mutation strings) of the disclosure. Accordingly, provided here are chymase-based engineered proteases, comprising one or more modifications (mutation strings) provided in Table 7A. Such exemplary engineered proteases may be capable of cleaving Factor B, or display other cleavage activity. As examples, engineered chymase-based engineered proteases include proteases having the exemplary combination of modifications: C22S/P38Q/K40M/F41R/V138I/F173Y/D175R/A190S/V213A/S218V/A226R, and C22S/P38Q/K40M/F41H/V138I/F173Y/D175R/A190S/V213A/S218V/A226R, based on chymotrypsin numbering.

Table 7B: Exemplary Chymase-Based Engineered Proteases

Chymotrypsin No.	Conventional No. (SEQ ID NO. 6)
C22S	C7S
C22S/A226R	C7S/A207R
C22S/L99H/F173L/K192R/Y215H/R217T/S218V/A226R	C7S/L86H/F157L/K179R/Y198H/R200T/S201V/A207R
C22S/L99H/F173I/K192R/Y215K/R217T/S218I/A226R	C7S/L86H/F157I/K179R/Y198K/R200T/S201I/A207R
C22S/L99H/F173L/K192R/Y215H/R217T/S218V/A226R	C7S/L86H/F157L/K179R/Y198H/R200T/S201V/A207R
C22S/F41I/L99W/G151L/F173K/R217S/A226R	C7S/F29I/L86W/G137L/F157K/R200S/A207R
C22S/F41R/L99G/G151T/F173Y/Y215R/R217M/A226R	C7S/F29R/L86G/G137T/F157Y/Y198R/R200M/A207R
C22S/F41R/L99H/G151T/F173Y/Y215K/R217M/A226R	C7S/F29R/L86H/G137T/F157Y/Y198K/R200M/A207R
C22S/F41R/L99Y/G151T/F173K/R217S/A226R	C7S/F29R/L86Y/G137T/F157K/R200S/A207R
C22S/F41V/L99F/G151R/F173K/R217S/A226R	C7S/F29V/L86F/G137R/F157K/R200S/A207R
C22S/F41V/L99H/G151R/F173L/Y215R/R217T/A226R	C7S/F29V/L86H/G137R/F157L/Y198R/R200T/A207R
F41V/L99H/G151V/F173Y/Y215K/R217G/A226R	F29V/L86H/G137V/F157Y/Y198K/R200G/A207R
C22S/F41V/L99W/G151L/F173K/R217S/A226R	C7S/F29V/L86W/G137L/F157K/R200S/A207R
C22S/K192R/A226R	C7S/K179R/A207R
C22S/L99R/F173L/K192R/Y215H/R217T/S218V/A226R	C7S/L86R/F157L/K179R/Y198H/R200T/S201V/A207R
C22S/L99Y/F173L/K192R/Y215H/R217T/S218V/A226R	C7S/L86Y/F157L/K179R/Y198H/R200T/S201V/A207R
C22S/L99H/F173Y/K192R/S218L/A226R	C7S/L86H/F157Y/K179R/S201L/A207R
C22S/L99R/F173Y/K192R/S218L/A226R	C7S/L86R/F157Y/K179R/S201L/A207R
C22S/L99Y/F173Y/K192R/S218L/A226R	C7S/L86Y/F157Y/K179R/S201L/A207R
C22S/K40L/F41A/K192R/A226R	C7S/K28L/F29A/K179R/A207R
C22S/K40L/F41G/K192R/A226R	C7S/K28L/F29G/K179R/A207R
C22S/K40M/F41V/K192R/A226R	C7S/K28L/F29V/K179R/A207R

Chymotrypsin No.	Conventional No. (SEQ ID NO. 6)
C22S/K40M/F41V/G151R/K192R/A226R	C7S/K28L/F29V/G137R/K179R/A207R
C22S/K40L/F41V/G151R/K192R/A226R	C7S/K28L/F29V/G137R/K179R/A207R
C22S/L99H/F173Y/K192R/Y215K/R217S/S218V/A226R	C7S/L86H/F157Y/K179R/Y198K/R200S/S201V/A207R
C22S/L99F/F173K/K192R/R217S/S218I/A226R	C7S/L86F/F157K/K179R/R200S/S201I/A207R
C22S/L99W/F173K/K192R/R217S/S218V/A226R	C7S/L86W/F157K/K179R/R200S/S201V/A207R
C22S/L99W/F173K/R217S/S218R/A226R	C7S/L86W/F157K/R200S/S201R/A207R
C22S/L99Q/F173L/Y215K/R217T/S218L/A226R	C7S/L86Q/F157L/Y198K/R200T/S201L/A207R
C22S/F41R/L99H/G151S/F173Y/Y215K/R217M/A226R	C7S/F29R/L86H/G137S/F157Y/Y198K/R200M/A207R
C22S/F41I/L99H/G151K/F173Y/Y215K/R217G/A226R	C7S/F29I/L86H/G137K/F157Y/Y198K/R200G/A207R
C22S/F41V/L99H/G151R/F173S/Y215K/A226R	C7S/F29V/L86H/G137R/F157S/Y198K/A207R
C22S/F41R/L99H/F173Y/Y215K/R217M/A226R	C7S/F29R/L86H/F157Y/Y198K/R200M/A207R
C22S/F41K/L99H/G151A/F173Y/Y215K/R217S/A226R	C7S/F29K/L86H/G137A/F157Y/Y198K/R200S/A207R
C22S/F41R/L99H/G151A/F173S/Y215K/R217T/A226R	C7S/F29R/L86H/G137A/F157S/Y198K/R200T/A207R
C22S/F41L/L99H/G151T/F173Y/Y215K/A226R	C7S/F29L/L86H/G137T/F157Y/Y198K/A207R
C22S/F41R/L99H/G151T/F173Y/Y215K/A226R	C7S/F29R/L86H/G137T/F157Y/Y198K/A207R
C22S/F41G/L99H/G151T/F173Y/Y215K/A226R	C7S/F29G/L86H/G137T/F157Y/Y198K/A207R
C22S/F41R/L99H/F173Y/Y215K/A226R	C7S/F29R/L86H/F157Y/Y198K/A207R
C22S/F41V/L99H/F173Y/Y215K/A226R	C7S/F29V/L86H/F157Y/Y198K/A207R
C22S/F41G/L99H/F173Y/Y215K/A226R	C7S/F29G/L86H/F157Y/Y198K/A207R
C22S/F41R/L99H/G151T/F173L/Y215K/A226R	C7S/F29R/L86H/G137T/F157L/Y198K/A207R
C22S/F41V/L99H/G151T/F173L/Y215K/A226R	C7S/F29V/L86H/G137T/F157L/Y198K/A207R
C22S/F41G/L99H/G151T/F173L/Y215K/A226R	C7S/F29G/L86H/G137T/F157L/Y198K/A207R
C22S/F41R/L99H/F173L/Y215K/A226R	C7S/F29R/L86H/F157L/Y198K/A207R

Chymotrypsin No.	Conventional No. (SEQ ID NO. 6)
C22S/F41V/L99H/G151T/F173Y/Y215K/A226R	C7S/F29V/L86H/G137T/F157Y/Y198K/A207R
C22S/F41L/L99H/F173Y/Y215K/A226R	C7S/F29L/L86H/F157Y/Y198K/A207R
C22S/F41L/L99H/G151T/F173L/Y215K/A226R	C7S/F29L/L86H/G137T/F157L/Y198K/A207R
C22S/F41L/L99H/F173L/Y215K/A226R	C7S/F29L/L86H/F157L/Y198K/A207R
C22S/F41V/L99H/F173L/Y215K/A226R	C7S/F29V/L86H/F157L/Y198K/A207R
C22S/F41G/L99H/F173L/Y215K/A226R	C7S/F29G/L86H/F157L/Y198K/A207R
C22S/L99R/K192R/S218L/A226R	C7S/L86R/K179R/S201L/A207R
C22S/L99R/F173Y/S218L/A226R	C7S/L86R/F157Y/S201L/A207R
C22S/L99R/F173Y/K192R/A226R	C7S/L86R/F157Y/K179R/A207R
C22S/K93Q/G119D/F173S/K192R/A226R	C7S/K80Q/G106D/F157S/K179R/A207R
C22S/P38Q/H100P/L116R/F123L/Q180H/S218I/A226R/R235S	C7S/P26Q/H87P/L103R/F110L/Q165H/S201I/A207R/R216S
E21G/C22S/R61H/M135T/F173Y/D175N/A209V/A226R	E6G/C7S/R49H/M122T/F157Y/D159N/A192V/A207R
C22S/K111E/F173L/Y215H/A226R	C7S/K98E/F157L/Y198H/A207R
C22S/F173L/Y215H/A226R	C7S/F157L/Y198H/A207R
C22S/M135T/P150S/S152G/Q166H/D175N/A190T/A226R	C7S/M122T/P136S/S138G/Q152H/D159N/A177T/A207R
C22S/P38Q/H100P/L116R/F123L/Q180H/A226R/R235S	C7S/P26Q/H87P/L103R/F110L/Q165H/A207R/R216S
C22S/V138I/L160V/S189T/A226R	C7S/V125I/L146V/S176T/A207R
C22S/V138L/A226R	C7S/V125L/A207R
C22S/V138L/A226R	C7S/V125L/A207R
C22S/L160V/S189T/A226R	C7S/L146V/S176T/A207R
C22S/S189T/A226R	C7S/S176T/A207R
C22S/S218I/A226R	C7S/S201I/A207R
C22S/F173Y/A226R	C7S/F157Y/A207R
C22S/D175N/A226R	C7S/D159N/A207R
C22S/F173Y/D175N/A226R	C7S/F157Y/D159N/A207R
C22S/F173Y/D175N/A226R	C7S/F157Y/D159N/A207R
C22S/A220S/A226R	C7S/A203S/A207R
C22S/S189T/A190S/F191S/A220L/A226R	C7S/S176T/A177S/A178S/A203L/A207R

Chymotrypsin No.	Conventional No. (SEQ ID NO. 6)
C22S/K40L/F41R/L99N/F173Y/S218L/A226R	C7S/K28L/F29R/L86N/F157Y/S201L/A207R
C22S/K40M/F41R/L99H/G151P/F173S/S218V/A226R	C7S/K28L/F29R/L86H/G137P/F157S/S201V/A207R
C22S/K40M/F41R/L99H/G151A/F173S/S218V/A226R	C7S/K28L/F29R/L86H/G137A/F157S/S201V/A207R
C22S/K40M/F41I/L99H/G151R/F173S/S218L/A226R	C7S/K28L/F29I/L86H/G137R/F157S/S201L/A207R
C22S/K40M/F41R/L99N/G151S/F173Y/S218L/A226R	C7S/K28L/F29R/L86N/G137S/F157Y/S201L/A207R
C22S/K40M/F41R/L99N/G151P/F173S/S218V/A226R	C7S/K28L/F29R/L86N/G137P/F157S/S201V/A207R
C22S/K40M/F41R/L99H/G151P/F173S/S218L/A226R	C7S/K28L/F29R/L86H/G137P/F157S/S201I/A207R
C22S/K40L/F41H/L99N/F173Y/S218L/A226R	C7S/K28L/F29H/L86N/F157Y/S201L/A207R
C22S/K40M/F41V/L99N/G151R/F173S/S218V/A226R	C7S/K28L/F29V/L86N/G137R/F157S/S201V/A207R
C22S/K40M/F41I/L99N/G151R/F173S/S218V/A226R	C7S/K28L/F29I/L86N/G137R/F157S/S201V/A207R
C22S/F41I/L99H/G151R/F173S/S218L/A226R	C7S/F29I/L86H/G137R/F157S/S201L/A207R
C22S/K40L/F41L/L99N/G151H/F173Y/S218V/A226R	C7S/K28L/F29L/L86N/G137H/F157Y/S201V/A207R
C22S/K40L/F41K/L99H/G151S/F173Y/S218L/A226R	C7S/K28L/F29K/L86H/G137S/F157Y/S201L/A207R
C22S/K40L/F41K/L99H/F173Y/S218L/A226R	C7S/K28L/F29K/L86H/F157Y/S201L/A207R
C22S/K40L/F41K/L99N/F173Y/S218L/A226R	C7S/K28L/F29K/L86N/F157Y/S201L/A207R
C22S/K40M/F41V/L99N/G151R/F173S/S218E/A226R	C7S/K28L/F29V/L86N/G137R/F157S/S201E/A207R
C22S/A190S/A226R	C7S/A177S/A207R
C22S/S189T/A190S/A226R	C7S/S176T/A177S/A207R
C22S/K40L/F41R/L99N/F173Y/D175N/S218L/A226R	C7S/K28L/F29R/L86N/F157Y/D159N/S201L/A207R
C22S/K40L/F41H/L99N/F173Y/D175N/S218L/A226R	C7S/K28L/F29H/L86N/F157Y/D159N/S201L/A207R

Chymotrypsin No.	Conventional No. (SEQ ID NO. 6)
C22S/K40L/F41K/L99N/F173Y/D175N/S218L/A226R	C7S/K28L/F29K/L86N/F157Y/D159N/S201L/A207R
C22S/P38Q/V138I/V183I/L199V/A226R/F228V	C7S/P26Q/V125I/V168I/L186V/A207R/F209V
C22S/V138T/A190S/V213A/A226R	C7S/V125T/A177S/V196A/A207R
C22S/P38Q/V138L/A190G/L199V/V213A/A226R/F228A	C7S/P26Q/V125L/A177G/L186V/V196A/A207R/F209A
C22S/P38Q/V138I/A226R/F228A	C7S/P26Q/V125I/A207R/F209A
C22S/F41K/L99N/F173Y/D175N/S218L/A226R	C7S/F29K/L86N/F157Y/D159N/S201L/A207R
C22S/K40L/L99N/F173Y/D175N/S218L/A226R	C7S/K28L/L86N/F157Y/D159N/S201L/A207R
C22S/K40L/F41K/F173Y/D175N/S218L/A226R	C7S/K28L/F29K/F157Y/D159N/S201L/A207R
C22S/K40L/F41K/L99N/D175N/S218L/A226R	C7S/K28L/F29K/L86N/D159N/S201L/A207R
C22S/K40L/F41K/L99N/F173Y/D175N/A226R	C7S/K28L/F29K/L86N/F157Y/D159N/A207R
C22S/V138A/G145D/S189T/A190S/A226R	C7S/V125A/G132D/S176T/A177S/A207R
C22S/A226R/F228A	C7S/A207R/F209A
C22S/V138A/S189T/A226R/F228M	C7S/V125A/S176T/A207R/F209M
C22S/V138L/S189T/A190G/A226R	C7S/V125L/S176T/A177G/A207R
C22S/V138S/S189T/A226R/F228M	C7S/V125S/S176T/A207R/F209M
C22S/S189T/A226R/F228M	C7S/S176T/A207R/F209M
C22S/V138A/A226R	C7S/V125A/A207R
C22S/V138A/S189T/A226R	C7S/V125A/S176T/A207R
C22S/K40M/F41R/L99N/F173Y/D175S/S218L/A226R	C7S/K28L/F29R/L86N/F157Y/D159S/S201L/A207R
C22S/K40M/F41R/L99H/F173Y/D175W/S218L/A226R	C7S/K28L/F29R/L86H/F157Y/D159W/S201L/A207R
C22S/K40M/F41K/F173Y/D175R/S218E/A226R	C7S/K28L/F29K/F157Y/D159R/S201E/A207R
C22S/K40M/F41K/L99N/F173Y/D175R/S218T/A226R	C7S/K28L/F29K/L86N/F157Y/D159R/S201T/A207R
C22S/K40L/F41K/L99Q/F173Y/D175N/S218V/A226R	C7S/K28L/F29K/L86Q/F157Y/D159N/S201V/A207R
C22S/K40M/F41R/L99Q/F173Y/D175R/S218V/A226R	C7S/K28L/F29R/L86Q/F157Y/D159R/S201V/A207R

Chymotrypsin No.	Conventional No. (SEQ ID NO. 6)
C22S/K40L/F41K/L99H/F173Y/D175S/S218I/A226R	C7S/K28L/F29K/L86H/F157Y/D159S/S201I/A207R
C22S/K40L/F41R/L99G/F173Y/D175R/S218V/A226R	C7S/K28L/F29R/L86G/F157Y/D159R/S201V/A207R
C22S/K40L/F41R/L99H/F173Y/D175Y/S218I/A226R	C7S/K28L/F29R/L86H/F157Y/D159Y/S201I/A207R
C22S/K40M/F41R/L99Q/F173Y/D175R/S218I/A226R	C7S/K28L/F29R/L86Q/F157Y/D159R/S201I/A207R
C22S/P38Q/V138I/A190S/V213A/A226R	C7S/P26Q/V125I/A177S/V196A/A207R
C22S/P38Q/V213Q/A226R/F228A	C7S/P26Q/V196Q/A207R/F209A
C22S/P38Q/K40A/F41R/L99H/F173Y/D175Y/V213Q/S218V/A226R/F228A	C7S/P26Q/K28A/F29R/L86H/F157Y/D159Y/V196Q/S201V/A207R/F209A
C22S/P38Q/K40A/F41R/L99H/D175N/V213Q/S218I/A226R/F228A	C7S/P26Q/K28A/F29R/L86H/D159N/V196Q/S201I/A207R/F209A
C22S/P38Q/K40M/F41R/L99M/F173Y/D175N/V213Q/S218V/A226R/F228A	C7S/P26Q/K28L/F29R/L86M/F157Y/D159N/V196Q/S201V/A207R/F209A
C22S/P38Q/K40M/F41R/D175N/V213Q/S218V/A226R/F228A	C7S/P26Q/K28L/F29R/D159N/V196Q/S201V/A207R/F209A
C22S/P38Q/K40L/F41R/V138I/F173Y/D175N/A190S/V213A/S218V/A226R	C7S/P26Q/K28L/F29R/V125I/F157Y/D159N/A177S/V196A/S201V/A207R
C22S/P38Q/K40L/F41R/L99H/V138I/F173Y/D175S/A190S/V213A/S218I/A226R	C7S/P26Q/K28L/F29R/L86H/V125I/F157Y/D159S/A177S/V196A/S201I/A207R
C22S/P38Q/K40M/F41R/L99F/V138I/F173S/D175H/A190S/V213A/S218V/A226R	C7S/P26Q/K28L/F29R/L86F/V125I/F157S/D159H/A177S/V196A/S201V/A207R
C22S/P38Q/K40A/F41R/L99H/V138I/F173Y/D175N/A190S/V213A/S218V/A226R	C7S/P26Q/K28A/F29R/L86H/V125I/F157Y/D159N/A177S/V196A/S201V/A207R
C22S/P38Q/K40A/F41K/L99H/V138I/D175S/A190S/V213A/S218V/A226R	C7S/P26Q/K28A/F29K/L86H/V125I/D159S/A177S/V196A/S201V/A207R
C22S/P38Q/K40M/F41R/L99N/V138I/F173Y/D175T/A190S/V213A/S218V/A226R	C7S/P26Q/K28L/F29R/L86N/V125I/F157Y/D159T/A177S/V196A/S201V/A207R
C22S/P38Q/K40M/F41R/V138I/F173Y/D175R/A190S/V213A/S218V/A226R	C7S/P26Q/K28L/F29R/V125I/F157Y/D159R/A177S/V196A/S201V/A207R

Chymotrypsin No.	Conventional No. (SEQ ID NO. 6)
C22S/P38Q/K40L/F41R/L99N/V138I/F173Y/D175N/A190S/V213A/S218V/A226R	C7S/P26Q/K28L/F29R/L86N/V125I/F157Y/D159N/A177S/V196A/S201V/A207R
C22S/P38Q/K40A/F41R/V138I/D175R/A190S/V213A/S218T/A226R	C7S/P26Q/K28A/F29R/V125I/D159R/A177S/V196A/S201T/A207R
C22S/P38Q/K40A/F41R/L99H/V138I/F173Y/A190S/V213A/S218V/A226R	C7S/P26Q/K28A/F29R/L86H/V125I/F157Y/A177S/V196A/S201V/A207R
C22S/P38Q/K40M/F41H/L99N/V138I/F173Y/D175S/A190S/V213A/S218T/A226R	C7S/P26Q/K28L/F29H/L86N/V125I/F157Y/D159S/A177S/V196A/S201T/A207R
C22S/P38Q/K40M/F41K/L99Y/V138I/F173M/D175Y/A190S/V213A/S218V/A226R	C7S/P26Q/K28L/F29K/L86Y/V125I/F157M/D159Y/A177S/V196A/S201V/A207R
C22S/P38Q/K40A/F41R/V138I/F173S/D175T/A190S/V213A/S218V/A226R	C7S/P26Q/K28A/F29R/V125I/F157S/D159T/A177S/V196A/S201V/A207R
C22S/P38Q/K40L/F41R/V138I/F173L/D175H/A190S/V213A/S218V/A226R	C7S/P26Q/K28L/F29R/V125I/F157L/D159H/A177S/V196A/S201V/A207R
C22S/P38Q/K40M/F41R/L99N/V138I/D175N/A190S/V213A/S218T/A226R	C7S/P26Q/K28L/F29R/L86N/V125I/D159N/A177S/V196A/S201T/A207R
C22S/P38Q/K40M/F41H/V138I/F173Y/D175R/A190S/V213A/S218V/A226R	C7S/P26Q/K28L/F29H/V125I/F157Y/D159R/A177S/V196A/S201V/A207R
C22S/P38Q/K40M/F41R/L99Q/V138I/F173Y/D175A/A190S/V213A/S218T/A226R	C7S/P26Q/K28L/F29R/L86Q/V125I/F157Y/D159A/A177S/V196A/S201T/A207R
C22S/P38Q/K40A/F41R/L99H/V138I/D175R/A190S/V213A/S218L/A226R	C7S/P26Q/K28A/F29R/L86H/V125I/D159R/A177S/V196A/S201L/A207R
C22S/P38Q/K40M/F41R/L99F/V138I/F173L/D175N/A190S/V213A/A226R	C7S/P26Q/K28L/F29R/L86F/V125I/F157L/D159N/A177S/V196A/A207R
C22S/P38Q/K40M/F41R/L99Y/V138I/F173L/D175S/A190S/V213A/S218V/A226R	C7S/P26Q/K28L/F29R/L86Y/V125I/F157L/D159S/A177S/V196A/S201V/A207R
C22S/P38Q/K40L/F41R/V138I/D175N/A190S/V213A/S218V/A226R	C7S/P26Q/K28L/F29R/V125I/D159N/A177S/V196A/S201V/A207R
C22S/P38Q/K40M/F41R/L99Y/V138I/F173Y/D175S/A190S/V213A/S218V/A226R	C7S/P26Q/K28L/F29R/L86Y/V125I/F157Y/D159S/A177S/V196A/S201V/A207R
C22S/P38Q/K40L/F41R/V138I/F173M/D175N/A190S/V213A/S218L/A226R	C7S/P26Q/K28L/F29R/V125I/F157M/D159N/A177S/V196A/S201L/A207R

Chymotrypsin No.	Conventional No. (SEQ ID NO. 6)
C22S/P38Q/K40L/F41R/L99N/V138I/F173Y/D175T/A190S/V213A/S218V/A226R	C7S/P26Q/K28L/F29R/L86N/V125I/F157Y/D159T/A177S/V196A/S201V/A207R
C22S/P38Q/K40M/F41R/V138I/F173L/D175N/A190S/V213A/S218L/A226R	C7S/P26Q/K28L/F29R/V125I/F157L/D159N/A177S/V196A/S201L/A207R
C22S/P38Q/K40L/F41K/L99N/V138I/F173Y/D175S/A190S/V213A/S218I/A226R	C7S/P26Q/K28L/F29K/L86N/V125I/F157Y/D159S/A177S/V196A/S201I/A207R
C22S/P38Q/K40A/F41R/L99Y/V138I/F173L/D175R/A190S/V213A/S218L/A226R	C7S/P26Q/K28A/F29R/L86Y/V125I/F157L/D159R/A177S/V196A/S201L/A207R
C22S/P38Q/K40L/F41R/L99Q/V138I/F173Y/D175N/A190S/V213A/S218V/A226R	C7S/P26Q/K28L/F29R/L86Q/V125I/F157Y/D159N/A177S/V196A/S201V/A207R
C22S/P38Q/K40M/F41R/L99Q/V138I/F173Y/D175N/A190S/V213A/S218V/A226R	C7S/P26Q/K28L/F29R/L86Q/V125I/F157Y/D159N/A177S/V196A/S201V/A207R
C22S/P38Q/K40V/F41R/V138I/F173L/D175R/A190S/V213A/S218I/A226R	C7S/P26Q/K28V/F29R/V125I/F157L/D159R/A177S/V196A/S201I/A207R
C22S/P38Q/K40M/F41T/V138I/D175R/A190S/V213A/S218I/A226R	C7S/P26Q/K28L/F29T/V125I/D159R/A177S/V196A/S201I/A207R
C22S/P38Q/K40M/F41R/V138I/F173L/D175K/A190S/V213A/S218V/A226R	C7S/P26Q/K28L/F29R/V125I/F157L/D159K/A177S/V196A/S201V/A207R
C22S/P38Q/K40M/F41R/L99Y/V138I/F173S/D175S/A190S/V213A/S218T/A226R	C7S/P26Q/K28L/F29R/L86Y/V125I/F157S/D159S/A177S/V196A/S201T/A207R
C22S/P38Q/K40A/F41T/L99Y/V138I/F173S/D175H/A190S/V213A/S218I/A226R	C7S/P26Q/K28A/F29T/L86Y/V125I/F157S/D159H/A177S/V196A/S201I/A207R
C22S/P38Q/K40A/F41R/V138I/F173L/D175R/A190S/V213A/S218L/A226R	C7S/P26Q/K28A/F29R/V125I/F157L/D159R/A177S/V196A/S201L/A207R
C22S/P38Q/K40M/F41R/L99Y/V138I/F173L/D175Q/A190S/V213A/S218T/A226R	C7S/P26Q/K28L/F29R/L86Y/V125I/F157L/D159Q/A177S/V196A/S201T/A207R
C22S/P38Q/K40A/F41Q/V138I/F173S/D175W/A190S/V213A/S218V/A226R	C7S/P26Q/K28A/F29Q/V125I/F157S/D159W/A177S/V196A/S201V/A207R
C22S/P38Q/K40M/F41H/V138I/D175S/A190S/V213A/A226R	C7S/P26Q/K28L/F29H/V125I/D159S/A177S/V196A/A207R
C22S/P38Q/K40V/F41R/V138I/F173L/D175N/A190S/V213A/S218V/A226R	C7S/P26Q/K28V/F29R/V125I/F157L/D159N/A177S/V196A/S201V/A207R

Chymotrypsin No.	Conventional No. (SEQ ID NO. 6)
C22S/P38Q/K40V/F41R/L99H/V138I/F173M/D175N/A190S/V213A/S218T/A226R	C7S/P26Q/K28V/F29R/L86H/V125I/F157M/D159N/A177S/V196A/S201T/A207R
C22S/P38Q/K40L/F41R/V138I/F173L/D175R/A190S/V213A/S218V/A226R	C7S/P26Q/K28L/F29R/V125I/F157L/D159R/A177S/V196A/S201V/A207R
C22S/P38Q/K40L/F41R/L99M/V138I/F173L/D175R/A190S/V213A/S218V/A226R	C7S/P26Q/K28L/F29R/L86M/V125I/F157L/D159R/A177S/V196A/S201V/A207R
C22S/P38Q/K40A/F41R/L99H/V138I/F173M/D175T/A190S/V213A/S218I/A226R	C7S/P26Q/K28A/F29R/L86H/V125I/F157M/D159T/A177S/V196A/S201I/A207R
C22S/P38Q/K40M/F41R/L99Y/V138I/F173L/D175S/A190S/V213A/S218T/A226R	C7S/P26Q/K28L/F29R/L86Y/V125I/F157L/D159S/A177S/V196A/S201T/A207R
C22S/P38Q/K40A/F41R/L99H/V138I/F173Y/D175N/A190S/V213A/S218I/A226R	C7S/P26Q/K28A/F29R/L86H/V125I/F157Y/D159N/A177S/V196A/S201I/A207R
C22S/P38Q/K40M/F41R/L99W/V138I/F173M/D175H/A190S/V213A/S218T/A226R	C7S/P26Q/K28L/F29R/L86W/V125I/F157M/D159H/A177S/V196A/S201T/A207R
C22S/P38Q/K40M/F41H/L99H/V138I/D175S/A190S/V213A/S218V/A226R	C7S/P26Q/K28L/F29H/L86H/V125I/D159S/A177S/V196A/S201V/A207R
C22S/P38Q/K40V/F41R/V138I/F173S/D175N/A190S/V213A/S218I/A226R	C7S/P26Q/K28V/F29R/V125I/F157S/D159N/A177S/V196A/S201I/A207R
C22S/P38Q/K40L/F41R/L99F/V138I/F173L/D175N/A190S/V213A/A226R	C7S/P26Q/K28L/F29R/L86F/V125I/F157L/D159N/A177S/V196A/A207R
C22S/P38Q/K40M/F41R/L99H/V138I/D175Q/A190S/V213A/S218V/A226R	C7S/P26Q/K28L/F29R/L86H/V125I/D159Q/A177S/V196A/S201V/A207R
C22S/P38Q/K40L/F41R/V138I/F173Y/D175H/A190S/V213A/S218T/A226R	C7S/P26Q/K28L/F29R/V125I/F157Y/D159H/A177S/V196A/S201T/A207R
C22S/P38Q/K40M/F41K/L99W/V138I/F173M/A190S/V213A/S218N/A226R	C7S/P26Q/K28L/F29K/L86W/V125I/F157M/A177S/V196A/S201N/A207R
C22S/P38Q/K40Q/F41R/V138I/F173Y/D175N/A190S/V213A/S218V/A226R	C7S/P26Q/K28Q/F29R/V125I/F157Y/D159N/A177S/V196A/S201V/A207R
C22S/P38Q/K40I/F41R/L99N/V138I/D175T/A190S/V213A/S218V/A226R	C7S/P26Q/K28I/F29R/L86N/V125I/D159T/A177S/V196A/S201V/A207R
C22S/P38Q/K40Q/F41R/L99H/V138I/F173Y/D175S/A190S/V213A/S218V/A226R	C7S/P26Q/K28Q/F29R/L86H/V125I/F157Y/D159S/A177S/V196A/S201V/A207R

Chymotrypsin No.	Conventional No. (SEQ ID NO. 6)
C22S/P38Q/K40A/F41R/L99H/V138I/F173Y/D175N/A190S/V213A/S218L/A226R	C7S/P26Q/K28A/F29R/L86H/V125I/F157Y/D159N/A177S/V196A/S201L/A207R
C22S/P38Q/K40M/F41R/L99N/V138I/F173Y/D175F/A190S/V213A/S218V/A226R	C7S/P26Q/K28L/F29R/L86N/V125I/F157Y/D159F/A177S/V196A/S201V/A207R
C22S/P38Q/K40M/F41R/L99N/V138I/F173Y/D175W/A190S/V213A/S218T/A226R	C7S/P26Q/K28L/F29R/L86N/V125I/F157Y/D159W/A177S/V196A/S201T/A207R
C22S/P38Q/K40L/F41R/L99Y/V138I/F173Y/D175N/A190S/V213A/S218V/A226R	C7S/P26Q/K28L/F29R/L86Y/V125I/F157Y/D159N/A177S/V196A/S201V/A207R
C22S/P38Q/K40M/F41R/L99S/V138I/F173Y/D175N/A190S/V213A/S218V/A226R	C7S/P26Q/K28L/F29R/L86S/V125I/F157Y/D159N/A177S/V196A/S201V/A207R
C22S/P38Q/K40A/F41R/L99H/V138I/D175N/A190S/V213A/S218I/A226R	C7S/P26Q/K28A/F29R/L86H/V125I/D159N/A177S/V196A/S201I/A207R
C22S/P38Q/K40M/F41R/V138I/F173S/D175Y/A190S/V213A/S218V/A226R	C7S/P26Q/K28L/F29R/V125I/F157S/D159Y/A177S/V196A/S201V/A207R
C22S/P38Q/K40M/F41R/V138I/F173L/D175R/A190S/V213A/S218T/A226R	C7S/P26Q/K28L/F29R/V125I/F157L/D159R/A177S/V196A/S201T/A207R
C22S/P38Q/K40L/F41R/V138I/F173Y/D175R/A190S/V213A/S218T/A226R	C7S/P26Q/K28L/F29R/V125I/F157Y/D159R/A177S/V196A/S201T/A207R
C22S/P38Q/K40A/F41K/L99N/V138I/D175H/A190S/V213A/S218V/A226R	C7S/P26Q/K28A/F29K/L86N/V125I/D159H/A177S/V196A/S201V/A207R
C22S/P38Q/K40M/F41R/V138I/F173Y/D175Y/A190S/V213A/S218I/A226R	C7S/P26Q/K28L/F29R/V125I/F157Y/D159Y/A177S/V196A/S201I/A207R

[0122] In some embodiments, a chymase-based engineered protease of the disclosure comprises at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 95% sequence identity to SEQ ID NO: 6.

[0123] In some embodiments, a chymase-based engineered protease of the disclosure comprises at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at

least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 95% sequence identity to SEQ ID NO: 6.

[0124] In some embodiments, a chymase -based engineered protease of the disclosure comprises a protease domain comprising at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 95% sequence identity to SEQ ID NO: 6.

KLK5-Based Engineered Proteases

[0125] In some embodiments, the engineered proteases are based on KLK5. In some embodiments, the engineered proteases are based on KLK5, and are specific for Factor B at a site that is targeted by Factor D, wherein the cleavage site comprises the amino acid sequence QQKR/KIV (SEQ ID NO: 9).

[0126] In some embodiments, the KLK5-based engineered proteases are based on a KLK5 comprising the amino acid sequence as set forth in SEQ ID NO: 23. Residues within or modifications to the KLK5 can be referred to by numbering the residues of KLK5 by chymotrypsin numbering. Presented in Table 7C is the chymotrypsin numbering scheme of amino acid positions 45-271 of KLK5 SEQ ID NO: 28, as set forth in SEQ ID NO: 23. Table 7C lists the amino acid residues 45-271 of SEQ ID NO: 23 above the amino acid single letter abbreviation, and the corresponding chymotrypsin numbering below each amino acid single letter abbreviation. A residue that exists in a protease that does not exist in a chymotrypsin is represented by a letter at the end of the notation. For example, residues in chymotrypsin at amino acid 36 based on chymotrypsin numbering which are inserted into an engineered KLK5 are referred to as 36a, 36b, 36c.

[0127] Table 7C provides the chymotrypsin numbering schema and its corresponding conventional numbering schema for the KLK5 protease domain. In subsequent tables, and throughout the disclosure, the modifications to the KLK5 protease domain are referred to either with chymotrypsin numbering, or using conventional amino acid numbering. If a particular modification is provided only with a chymotrypsin numbering notation, the skilled artisan will

understand how to refer to Table 7C and perform the necessary conversion to understand the modification in conventional amino acid terms, and vice versa.

Table 7C: Chymotrypsin Numbering of KLK5

Conv No. (SEQ ID 23)	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59
Amino Acid	I	I	N	G	S	D	C	D	M	H	T	Q	P	W	Q
Chymo. No.	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Conv No. (SEQ ID 23)	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74
Amino Acid	A	A	L	L	L	R	P	N	Q	L	Y	C	G	A	V
Chymo. No.	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
Conv No. (SEQ ID 23)	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89
Amino Acid	L	V	H	P	Q	W	L	L	T	A	A	H	C	R	K
Chymo. No.	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
Conv No. (SEQ ID 23)	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104
Amino Acid	K	V	F	R	V	R	L	G	H	Y	S	L	S	P	V
Chymo. No.	61	62	63	64	65	66	67	69	70	71	72	73	74	74A	75
Conv No. (SEQ ID 23)	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119
Amino Acid	Y	E	S	G	Q	Q	M	F	Q	G	V	K	S	I	P
Chymo. No.	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
Conv No. (SEQ ID 23)	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134
Amino Acid	H	P	G	Y	S	H	P	G	H	S	N	D	L	M	L
Chymo. No.	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105
Conv No. (SEQ ID 23)	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149
Amino Acid	I	K	L	N	R	R	I	R	P	T	K	D	V	R	P
Chymo. No.	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120
Conv No. (SEQ ID 23)	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164
Amino Acid	I	N	V	S	S	H	C	P	S	A	G	T	K	C	L
Chymo. No.	121	122	123	124	125	128	129	130	131	132	133	134	135	136	137

Conv No. (SEQ ID 23)	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179
Amino Acid	V	S	G	W	G	T	T	K	S	P	Q	V	H	F	P
Chymo. No.	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152
Conv No. (SEQ ID 23)	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194
Amino Acid	K	V	L	Q	C	L	N	I	S	V	L	S	Q	K	R
Chymo. No.	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167
Conv No. (SEQ ID 23)	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209
Amino Acid	C	E	D	A	Y	P	R	Q	I	D	D	T	M	F	C
Chymo. No.	168	169	170	171	172	173	174	174A	176	177	178	179	180	181	182
Conv No. (SEQ ID 23)	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224
Amino Acid	A	G	D	K	A	G	R	D	S	C	Q	G	D	S	G
Chymo. No.	183	184	185	186	186A	187	188	189	190	191	192	193	194	195	196
Conv No. (SEQ ID 23)	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239
Amino Acid	G	P	V	V	C	N	G	S	L	Q	G	L	V	S	W
Chymo. No.	197	198	199	200	201	202	203	204	209	210	211	212	213	214	215
Conv No. (SEQ ID 23)	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254
Amino Acid	G	D	Y	P	C	A	R	P	N	R	P	G	V	Y	T
Chymo. No.	216	217	218	219	220	221	221A	222	223	224	225	226	227	228	229
Conv No. (SEQ ID 23)	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269
Amino Acid	N	L	C	K	F	T	K	W	I	Q	E	T	I	Q	A
Chymo. No.	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244
Conv No. (SEQ ID 23)	270	271													
Amino Acid	N	S													
Chymo. No.	245	246													

[0128] It should be understood that the engineered proteases of the disclosure are not limited to those presented in the tables above. Such modifications may increase the half-life, bioavailability, or other characteristics of the serine proteases.

Fusion Proteins

[0129] The engineered proteases can be further modified, e.g. they can include the fusion (addition) of another component or domain. Examples of such components or domains include, but are not limited to, half-life extenders, and activation peptides or activation signals. For example, a component capable of increasing the half-life or bioavailability of an engineered protease of the disclosure can be added. A half-life extender can include, but is not limited to, Fc (e.g., IgG1, IgG2, IgG3, or IgG4), affibodies, PEG, and albumin such as Human Serum Albumin (HSA).

[0130] Such additions of the additional components can be added by, for example, PASylation®. Such additions can extend the half-life or bioavailability of the engineered protease compared to a serine protease that does not include the half-life extender. In some embodiments, the addition of a half-life extender or other similar component can also improve or alter any one or more properties of the engineered protease, including, but not limited to, stability, bioavailability, serum half-life, shelf-life, trafficking ability, and immunogenicity.

[0131] Accordingly, in some embodiments, the engineered proteases provided herein can further comprise a half-life extender. In some embodiments, the half-life extender is an addition at the N-terminus of the engineered protease. In some embodiments, the half-life extender is an addition at the C-terminus of the engineered protease. In some embodiments, the half-life extender is added directly to the serine protease. In some embodiments, the half-life extender is added to the serine protease via a linker or more than one linker. In some embodiments, the half-life extender is Fc and is a human wild type Fc domain, or a variant thereof. In some embodiments, the half-life extender is albumin, e.g. a human serum albumin, or a variant thereof.

[0132] In some embodiments, the engineered proteases provided herein may comprise more than one half-life extender. In some embodiments, each of the half-life extenders are additions at the N-terminus of the serine protease. In some embodiments, each of the half-life extenders are additions at the C-terminus of the serine protease. In some embodiments, one half-life extender is an addition at the N-terminus and the other half-life extender is an addition at the C-terminus of the engineered protease. In some embodiments, the half-life extender is Fc and is a human

wild type Fc domain, or a variant thereof. In some embodiments, the half-life extender is albumin, e.g. a human serum albumin, or a variant thereof.

[0133] In exemplary embodiments, a chymase-based engineered protease of the disclosure is fused to a wild type Fc domain or variant thereof. In exemplary embodiments, a chymase-based engineered protease of the disclosure is fused to a human serum albumin, or variant thereof.

[0134] In exemplary embodiments, a uPA-based engineered protease of the disclosure is fused to a wild type Fc domain or variant thereof. In exemplary embodiments, a uPA -based engineered protease of the disclosure is fused to a human serum albumin, or variant thereof.

[0135] In exemplary embodiments, a MTSP-1-based engineered protease of the disclosure is fused to a wild type Fc domain or variant thereof. In exemplary embodiments, a MTSP-1-based engineered protease of the disclosure is fused to a human serum albumin, or variant thereof.

[0136] In exemplary embodiments, a KLK5-based engineered protease of the disclosure is fused to a wild type Fc domain or variant thereof. In exemplary embodiments, a KLK5-based engineered protease of the disclosure is fused to a human serum albumin, or variant thereof.

[0137] The fusion proteins also can include an activation sequence so that the resulting fusion protein containing an engineered protease of the disclosure is in an active form, such as a two chain form. Activation sequences can contain or be modified to contain a cysteine, which can form a disulfide bond with a free Cys, such as C122, for example, in the modified u-PA polypeptide, whereby, upon activation, the resulting activated polypeptide comprises two chains. Exemplary activation sequences include a enterokinase activation sequence and a furin activation sequence, and modified forms thereof.

Activity of Engineered Proteases

[0138] In some embodiments, the engineered proteases of the disclosure cleave Factor B at a site not targeted by Factor D, or at a site targeted by Factor D, and the cleavage at such site results in a reduction of a function of Factor B or a Factor B fragment. In some embodiments, the function of Factor B or a Factor B fragment is an interaction with at least one complement component. In some embodiments, the function of Factor B or a Factor B fragment is an interaction with hydrolyzed soluble C3. In some embodiments, the function of Factor B or a

Factor B fragment is an interaction with C3b. In some embodiments, the C3b is a membrane-bound C3b. In some embodiments, cleavage at a non-Factor D site occurs when Factor B is not bound to C3b. In some embodiments, cleavage at a Factor D site occurs when Factor B is not bound to C3b. In some embodiments, cleavage at a non-Factor D site occurs when Factor B is bound to C3b (i.e., complexed with C3b).

[0139] In some embodiments, the engineered proteases provided herein can cleave other peptide substrates that are not Factor B, while also being capable of cleaving Factor B. In some embodiments, the cleavage activity for a non-Factor B peptide substrate is about equal to or less than cleavage activity for the Factor B site.

[0140] In some embodiments, the engineered proteases provided herein have a K_{cat}/K_m of about 100, about 200, about 300, about 400, about 500, about 600, about 700, about 800, about 900, about 1,000, about 1,000, about 1,100, about 1,200, about 1,300, about 1,400, about 1,500, about 1,600, about 1,700, about 1,800, or about 1,900 $M^{-1} s^{-1}$ for Factor B cleavage. In some embodiments, the K_{cat}/K_m is up to or greater than about $10e^8$. In some embodiments, the engineered proteases provided herein have a K_{cat}/K_m of about 10^3 to about $10^6 M^{-1} s^{-1}$ for Factor B cleavage.

[0141] In some embodiments, the engineered proteases provided herein have an EC_{50} for Factor B cleavage of about 1 nM to about 20 nM. In some embodiments, the engineered proteases provided herein have an EC_{50} for Factor B of less than about 20 nM. In some embodiments, the engineered proteases provided herein have an EC_{50} for Factor B cleavage of less than about 1 nM. In some embodiments, the engineered proteases provided herein have an EC_{50} for Factor B cleavage of about 5 nM to about 100 nM. In some embodiments, the engineered proteases provided herein have an EC_{50} for cleavage of Factor B of about 20, about 25, or about 60 nM. In some embodiments, the EC_{50} for cleavage of Factor B is about 20 nM. In some embodiments, the EC_{50} for Factor B cleavage is about 50 nM. In some embodiments, the engineered proteases provided herein have an EC_{50} for Factor B cleavage of about 1,000 nM to about 4,500 nM. In some embodiments, the engineered proteases provided herein have an EC_{50} for Factor B cleavage of about 1,000 nM, or about 2,000 nM, or about 3,000 nM, or about 4,000 nM, or about 5,000 nM.

[0142] In some embodiments, the engineered proteases provided herein have a catalytic lifetime in human plasma of over about 72 hours. In some embodiments, the engineered proteases provided herein have a catalytic lifetime in human plasma of about or greater than about 120 hours. In some embodiments, the engineered proteases provided herein have a catalytic lifetime in human plasma of about 120 hours or more and are useful for chronic indications. In some embodiments, the engineered proteases provided herein have a catalytic lifetime in human plasma of about 24 hours. In some embodiments, the engineered proteases provided herein have a catalytic lifetime in human plasma of about 24 hours or more and are useful for acute indications.

[0143] In some embodiments, the engineered proteases provided herein have catalytic activity for about 1 day, about 2 days, about 3 days, about 4 days, about 5 days, about 6 days, about 7 days, about 8 days, about 9 days, or about 10 days. In some embodiments, the catalytic activity is between about 10% and about 85% of the initially measured activity after about 7 days.

[0144] In some embodiments, the engineered proteases provided herein have an increased half-life compared to an MTSP-1 or MTSP-1 protease domain that is not modified. In some embodiments, the engineered proteases provided herein have an increased bioavailability compared to an MTSP-1 or MTSP-1 protease domain that is not modified. In some embodiments, the engineered proteases provided herein have an increased half-life compared to a uPA or uPA protease domain that is not modified. In some embodiments, the engineered proteases provided herein have an increased bioavailability compared to a uPA or uPA protease domain that is not modified. In some embodiments, the engineered proteases provided herein have an increased half-life compared to a chymase or chymase protease domain that is not modified. In some embodiments, the engineered proteases provided herein have an increased bioavailability compared to a chymase or chymase protease domain that is not modified. In some embodiments, the engineered proteases provided herein have an increased half-life compared to a KLK5 or KLK5 protease domain that is not modified. In some embodiments, the engineered proteases provided herein have an increased bioavailability compared to a KLK5 or KLK5 protease domain that is not modified.

[0145] In some embodiments, the engineered proteases provided herein are non-immunogenic.

[0146] In some embodiments, the engineered proteases provided herein are in a zymogen form. As used herein, the zymogen form refers to a full-length protease prior to cleavage into the mature form. In some embodiments, the engineered proteases provided herein are in an active form, also referred to as the mature form. In some embodiments, the zymogen form may be activated to the mature form in vivo (in situ) upon administration. In some embodiments the zymogen form is activated ex vivo (in vitro) prior to e.g. administration of the engineered protease.

[0147] In some embodiments, the engineered protease is in an activated form. In some embodiments, the engineered protease is activated by an enzyme, e.g. an enterokinase. In some embodiments, a chymase-based engineered protease of the disclosure is activated by an enterokinase. In some embodiments, the engineered protease is activated during recombinant production in a host cell. In some embodiments, the activation by an enzyme during production in a host cell is by overexpression of the enzyme, e.g. an enterokinase. In some embodiments, the engineered protease is activated after production and secretion by a host cell, optionally in the media.

Uses of Engineered Proteases

[0148] The engineered proteases of the disclosure may be used for modulating the complement system.

[0149] In some embodiments, the engineered proteases of the disclosure are capable of modulating the classical complement pathway. In some embodiments, the engineered proteases of the disclosure are capable of modulating the alternate complement pathway. In some embodiments, the engineered proteases of the disclosure are capable of modulating the lectin complement pathway. In some embodiments, the engineered proteases of the disclosure are capable of decreasing the amplification of the complement system.

[0150] In some embodiments, the engineered proteases of the disclosure are capable of reducing a function of Factor B or a Factor B fragment. As discussed herein, in some embodiments, the engineered proteases of the disclosure are capable of reducing generation of Factor B fragments Ba and/or Bb or producing Factor B fragments Ba and/or Bb that are functionally inactive.

[0151] Provided herein is a method of inactivating Factor B, comprising contacting the Factor B with any of the engineered proteases disclosed herein. In some embodiments, using such a method, complement activation is inhibited. In some embodiments, the classical pathway of the complement pathway is inhibited. In some embodiments, the alternate pathway of the complement pathway is inhibited. In some embodiments, the lectin pathway of the complement pathway is inhibited.

[0152] In some embodiments, the method is *in vitro*. In some embodiments, the method is *in vivo*.

[0153] The engineered proteases of the disclosure may be used for therapeutics in a subject. Accordingly, provided herein is a method of treating a disease or condition in a subject in need thereof, comprising administering to the subject any one of the engineered proteases of the disclosure. In some embodiments, the disease or condition is associated with dysregulated complement, accordingly, in some embodiments, the disease or condition involves complement dysregulation. In some embodiments, the treatment is a replacement therapy. In some embodiments, the treatment blocks complement activation. In some embodiments, the treatment modulates autoimmunity. In some embodiments, the treatment is for endothelial or kidney cell injury.

[0154] In some exemplary embodiments, the disease or condition is selected from lupus nephritis, C3 glomerulopathy (C3G), primary IgA nephropathy, kidney transplant ischemia and reperfusion (I/R) injury, antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV), sepsis, acute respiratory distress syndrome (ARDS), SARS-associated coronavirus (SARS-CoV), atypical hemolytic uremic syndrome (aHUS), membranous nephropathy (MN) and paroxysmal nocturnal hemoglobinuria (PNH).

[0155] In some embodiments, the engineered proteases provided herein are useful for treatment of inflammatory diseases or condition. In some embodiments, the engineered proteases provided herein are capable of reducing inflammatory cytokines. In some exemplary embodiments, the engineered proteases provided herein are efficacious in reducing inflammatory cytokines IL-2 and IL-6, and chemokine CXCL9 and are useful for the treatment of diseases such as ARDS.

[0156] In some embodiments, the engineered protease is administered to the subject subcutaneously. In some embodiments, the engineered protease is activated *in situ* at the site of a dysregulated complement component, or at the site of dysregulated pathophysiology. In some embodiments, the engineered protease is provided in a liquid stable formulation. The *in vivo* administration of the engineered proteases can be carried out intravenously, intramuscularly, subcutaneously, topically, orally, transdermally, intraperitoneally, intraorbitally, intrathecally, intraarterially, intraventricularly, intranasally, transmucosally, through implantation, or through inhalation. In some embodiments, the engineered proteases provided herein are administered with a mechanical device.

Pharmaceutical Compositions

[0157] The disclosure also provides pharmaceutical compositions comprising any one of the engineered proteases disclosed herein, and optionally a pharmaceutical acceptable excipient or carrier. In some embodiments, the pharmaceutical composition is sterile. The pharmaceutical compositions may be formulated to be compatible with their intended routes of administration. In some embodiments, the pharmaceutical compositions of the disclosure are suitable for administration to a human subject, or other non-human primate.

Kits and Articles of Manufacture

[0158] The disclosure also provides a kit or article of manufacture comprising any one of the engineered proteases disclosed herein, or any pharmaceutical composition disclosed herein. In some embodiments, the kits may further include instructional materials for carrying out any of the methods disclosed herein. In some embodiments, the kits may further include sterile containers or vials for holding the fusion constructs and/or pharmaceutical compositions disclosed herein. In some embodiments, the kits may further include sterile delivery devices for administering the fusion constructs and/or pharmaceutical compositions disclosed herein. In some embodiments, an article of manufacture comprises any pharmaceutical composition of the disclosure.

Production of Engineered Proteases

[0159] Provided herein are methods and compositions for generating engineered proteases. Accordingly, provided herein are nucleic acids and vectors encoding any of the engineered

proteases of the disclosure. Also provided are cells comprising one or more nucleic acids encoding an engineered protease of the disclosure.

[0160] The engineered proteases provided herein can be cloned or isolated using any available methods known in the art for cloning and isolating nucleic acid molecules. Such methods include PCR amplification of nucleic acids and screening of libraries, including nucleic acid hybridization screening, antibody-based screening and activity-based screening.

[0161] Provided herein are methods of generating an engineered protease of the disclosure using an expression system, e.g. the engineered protease of the disclosure may be expressed in bacterial (e.g. *E. coli*), yeast, insect, or mammalian cells (e.g. CHO cells, HEK cells). In specific embodiments, transformation of host cells with recombinant DNA molecules that incorporate the isolated an engineered protease protein gene, cDNA, or synthesized DNA sequence enables generation of multiple copies of the gene. Thus, the gene can be obtained in large quantities by growing transformants, isolating the recombinant DNA molecules from the transformants and, when necessary, retrieving the inserted gene from the isolated recombinant DNA. In some embodiments, the engineered protease is active upon production. In some embodiments, the engineered protease needs to be activated upon production. In such embodiments, an engineered protease is engineered in an activated state; the method comprising producing the engineered protease in a zymogen form in a bacterial, yeast, or mammalian host system, and subsequently activated.

Administration of Engineered Proteases

[0162] In some embodiments, provided herein are methods for administering an engineered protease of the disclosure by delivery of vectors/nucleic acids encoding the engineered protease. In some embodiments, the methods involve administration of recombinant vectors. In some embodiments, provided herein are engineered proteases for use in gene expression therapy using non-viral vectors. In other embodiments, provided herein are engineered proteases for use in gene expression therapy using viral vectors. In some embodiments, cells are engineered to express an engineered protease, such as by integrating an engineered protease encoding-nucleic acid into a genomic location, either operatively linked to regulatory sequences or such that it is placed operatively linked to regulatory sequences in a genomic location. In some embodiments,

such cells are then administered locally or systemically to a subject, such as a subject in need of treatment.

[0163] Methods for amplification of nucleic acids can be used to isolate nucleic acid molecules encoding an engineered protease, including for example, polymerase chain reaction (PCR) methods. A nucleic acid containing material can be used as a starting material from which an engineered protease-encoding nucleic acid molecule can be isolated. For example, DNA and mRNA preparations, cell extracts, tissue extracts (e.g. from liver), fluid samples (e.g. blood, serum, saliva), samples from healthy and/or diseased subjects can be used in amplification methods. Nucleic acid libraries also can be used as a source of starting material. Primers can be designed to amplify and modify an engineered protease-encoding molecule. For example, primers can be designed based on expressed sequences from which an engineered protease is generated.

[0164] Additional nucleotide sequences can be joined to an engineered protease-encoding nucleic acid molecule, including linker sequences containing restriction endonuclease sites for the purpose of cloning the synthetic gene into a vector, for example, a protein expression vector or a vector designed for the amplification of the core protein coding DNA sequences. Furthermore, additional nucleotide sequences specifying functional DNA elements can be operatively linked to an engineered protease-encoding nucleic acid molecule. Examples of such sequences include, but are not limited to, promoter sequences designed to facilitate intracellular protein expression, and secretion sequences designed to facilitate protein secretion. Additional nucleotide sequences such as sequences specifying protein binding regions also can be linked to an engineered protease -encoding nucleic acid molecules. Such regions include, but are not limited to, sequences to facilitate uptake of an engineered protease into specific target cells, or otherwise enhance the pharmacokinetics of the synthetic gene.

Enumerated Embodiments

[0165] The disclosure provides for the following sets of non-limiting enumerated embodiments.

Set I

[0166] Embodiment I-1. An engineered protease of the S1A serine protease family, wherein the engineered protease is specific for and is capable of cleaving Factor B.

[0167] Embodiment I-2. The engineered protease of embodiment I- 1, wherein cleavage of Factor B by the engineered protease generates one or more functionally inactive fragments.

[0168] Embodiment I-3. The engineered protease of any of embodiment I-2, wherein the one or more functionally inactive fragments are capable of reducing complement activation.

[0169] Embodiment I-4. The engineered protease of any one of embodiments 1-3, wherein cleavage of Factor B results in the generation of a Factor B fragment that is reduced in function or results in a Factor B that is reduced in function.

[0170] Embodiment I-5. The engineered protease of any one of embodiments 1-4, wherein the Factor B is a rodent Factor B.

[0171] Embodiment I-6. The engineered protease of any one of embodiments 1-4, wherein the Factor B is a non-human primate Factor B.

[0172] Embodiment I-7. The engineered protease of embodiment I-6, wherein the non-human primate is cynomolgus monkey.

[0173] Embodiment I-8. The engineered protease of any one of embodiments 1-4, wherein the Factor B is human Factor B.

[0174] Embodiment I-9. The engineered protease of embodiment I-8, wherein the Factor B comprises the amino acid sequence as set forth in SEQ ID NO: 1.

[0175] Embodiment I-10. The engineered protease of any one of embodiments 1-9, wherein cleavage of Factor B occurs at a site not targeted by Factor D.

[0176] Embodiment I-11. The engineered protease of embodiment I-10, wherein cleavage at the site generates at least two fragments that are not Ba and Bb.

[0177] Embodiment I-12. The engineered protease of any one of embodiments 1-11, wherein cleavage at the site results in a reduction of the generation of Factor B cleavage products Ba and Bb as compared to cleavage by Factor D.

[0178] Embodiment I-13. The engineered protease of any one of embodiments 1-9, wherein cleavage of Factor B occurs at a site that is targeted by Factor D.

[0179] Embodiment I-14. The engineered protease of any one of embodiments 1-13, wherein the site targeted by Factor D comprises QQKR/KIV (SEQ ID NO: 9).

[0180] Embodiment I-15. The engineered protease of embodiment I-10, wherein the Factor B cleavage site comprises a sequence selected from: WEHR/KGT (SEQ ID NO: 10), KNQKR/QKQ (SEQ ID NO: 11), DVFY/QMI (SEQ ID NO: 12), EGVD/AE (SEQ ID NO: 13), DHKL/KSG (SEQ ID NO: 14), TPW/SLA (SEQ ID NO: 15), KVSEAD (SEQ ID NO: 20), IRPSKG (SEQ ID NO: 4), GGEKRD (SEQ ID NO: 5), GKKEAG (SEQ ID NO: 3), and DHKL/KSG (SEQ ID NO: 21).

[0181] Embodiment I-16. The engineered protease of any one of embodiments 1-15, wherein the engineered protease is based on a chymotrypsin-like serine protease selected from the group consisting of: membrane type serine protease 1 (MTSP-1), urokinase-type plasminogen activator (uPA), chymase, and Kallikrein-related peptidase 5 (KLK5).

[0182] Embodiment I-17. The engineered protease of embodiment I-16, wherein the engineering of the engineered protease involves one or more modifications selected from the group consisting of substitution, addition, and deletion of an amino acid residue, and substitution, addition, and deletion of a domain of the chymotrypsin-like serine protease.

[0183] Embodiment I-18. The engineered protease of any one of embodiments 16-17, wherein the engineered protease is based on MTSP-1 or uPA, and the cleavage site comprises a sequence selected from: WEHR/KGT (SEQ ID NO: 10) and KNQKR/QKQ (SEQ ID NO: 11).

[0184] Embodiment I-19. The engineered protease of any one of embodiments 1-18, wherein the engineered protease is based on a MTSP-1.

[0185] Embodiment I-20. The engineered protease of any one of embodiments 1-18, wherein the engineered protease is not based on a MTSP-1.

[0186] Embodiment I-21. The engineered protease of embodiment I-19, comprising one or more modifications with respect to a MTSP-1 comprising an amino acid sequence as set forth in SEQ ID NO: 7, wherein the residues are numbered by chymotrypsin numbering.

[0187] Embodiment I-22. The engineered protease of any one of embodiments 1-18, wherein the engineered protease is based on a uPA.

[0188] Embodiment I-23. The engineered protease of any one of embodiments 1-18, wherein the engineered protease is not based on a uPA.

[0189] Embodiment I-24. The engineered protease of embodiment I-22, comprising one or more modifications with respect to a uPA comprising an amino acid sequence as set forth in SEQ ID NO: 8, wherein the residues are numbered by chymotrypsin numbering.

[0190] Embodiment I-25. The engineered protease of any one of embodiments 1-17, wherein the engineered protease is based on chymase.

[0191] Embodiment I-26. The engineered protease of embodiment I-25, wherein the engineered protease is based on chymase, and the cleavage site comprises a sequence selected from DVFY/QMI (SEQ ID NO: 12), EGVD/AE (SEQ ID NO: 13), DHKL/KSG (SEQ ID NO: 14), and TPW/SLA (SEQ ID NO: 15).

[0192] Embodiment I-27. The engineered protease of any one of embodiments I-1-17, wherein the engineered protease is based on KLK5.

[0193] Embodiment I-28. The engineered protease of embodiment I-25, comprising one or more modifications with respect to a chymase comprising an amino acid sequence as set forth in SEQ ID NO: 6, wherein the residues are numbered by chymotrypsin numbering.

[0194] Embodiment I-29. The engineered protease of embodiment I-19, wherein the one or more modifications is at one or more positions corresponding to one or more positions selected from D23, I41, L70, A77, F94, D96, F97, T98, F99, K110, C122, D125, Y146, Q175, V183, Q192, A204, D217, and K224 in a MTSP-1 comprising the sequence of amino acids set forth in SEQ ID NO: 7, wherein the residues are numbered by chymotrypsin numbering.

[0195] Embodiment I-30. The engineered protease of embodiment I-22, wherein the one or more modifications is at one or more positions corresponding to one or more positions selected from G18, R36, S37, V38, Y40, D60, A96, L97, A98, H99, C122, Y151, V159, A184, Q192, R217, K224 in a uPA comprising the sequence of amino acids set forth SEQ ID NO: 8, wherein the residues are numbered by chymotrypsin numbering.

[0196] Embodiment I-31. The engineered protease of embodiment I-25, wherein the one or more modifications is one or more positions corresponding to one or more positions selected from C22, S36, P38, G43, R49, K87, K93, I103, L114, L116, F123, V138, F173, D175, S189, A190, F191, K192, L199, V213, G216, A220, A226, F228 in a chymase comprising the sequence of amino acids set forth in SEQ ID NO: 6, wherein the residues are numbered by chymotrypsin numbering.

[0197] Embodiment I-32. The engineered protease of any one of embodiments 4-31, wherein the function of Factor B or a Factor B fragment is an interaction with at least one complement component.

[0198] Embodiment I-33. The engineered protease of any one of embodiments 4-31, wherein the function of Factor B or a Factor B fragment is an interaction with hydrolyzed soluble C3.

[0199] Embodiment I-34. The engineered protease of any one of embodiments 4-33, wherein the function of Factor B or a Factor B fragment is an interaction with C3b.

[0200] Embodiment I-35. The engineered protease of any one of embodiments 4-34, wherein the function of Factor B or a Factor B fragment is an interaction with membrane-bound C3b.

[0201] Embodiment I-36. The engineered protease of any one of embodiments 1-35, wherein cleavage occurs when Factor B is not bound to C3b.

[0202] Embodiment I-37. The engineered protease of any one of embodiments 1-36, wherein the cleavage activity for a non-Factor B peptide substrate is about equal to or less than cleavage activity for the Factor B site.

[0203] Embodiment I-38. The engineered protease of any one of embodiments 1-36, wherein the engineered protease has a kcat/Km of about 100, about 200, about 300, about 400, about 500, about 600, about 700, about 800, about 900, about 1,000, about 1,000, about 1,100, about 1,200,

about 1,300, about 1,400, about 1,500, about 1,600, about 1,700, about 1,800, or about 1,900 M⁻¹ s⁻¹ for Factor B cleavage.

[0204] Embodiment I-39. The engineered protease of any one of embodiments 1-38, wherein the engineered protease has a k_{cat}/K_m of about 103 to about 109 M⁻¹ s⁻¹ for Factor B cleavage.

[0205] Embodiment I-40. The engineered protease of any one of embodiments 1-39, wherein the engineered protease has an EC₅₀ for Factor B of less than about 20 nM.

[0206] Embodiment I-41. The engineered protease of any one of embodiments 1-40, wherein the engineered protease has an EC₅₀ for Factor B of less than about 1 nM.

[0207] Embodiment I-42. The engineered protease of any one of embodiments 1-39, wherein the engineered protease has an EC₅₀ for Factor B of about 20, about 25, or about 60 nM.

[0208] Embodiment I-43. The engineered protease of any one of embodiments 1-39, wherein the engineered protease has an EC₅₀ for cleaving Factor B of about 1,000 to about 4,500 nM.

[0209] Embodiment I-44. The engineered protease of any one of embodiments 1-43, wherein the engineered protease has a plasma half-life in human plasma of over about 72 hours.

[0210] Embodiment I-45. The engineered protease of any one of embodiments 1-44, wherein the engineered protease has a plasma half-life in human plasma of over about 120 hours.

[0211] Embodiment I-46. The engineered protease of any one of embodiments 1-45, wherein the engineered protease has a plasma half-life in human plasma of about 7 days.

[0212] Embodiment I-47. The engineered protease of embodiment I-46, wherein the catalytic activity is about 10% to about 50%, or about 90% to about 100%.

[0213] Embodiment I-48. The engineered protease of Embodiment I-29, wherein the engineered protease has an increased half-life compared to an MTSP-1 that is not modified.

[0214] Embodiment I-49. The engineered protease of any one of embodiments I-29 and I-48, wherein the engineered protease has an increased bioavailability compared to an MTSP-1 that is not modified.

- [0215] Embodiment I-50. The engineered protease of Embodiment I-30, wherein the engineered protease has an increased half-life compared to a uPA that is not modified.
- [0216] Embodiment I-51. The engineered protease of any one of embodiments I-30 and I-50, wherein the engineered protease has an increased bioavailability compared to a uPA that is not modified.
- [0217] Embodiment I-52. The engineered protease of Embodiment I-31, wherein the engineered protease has an increased half-life compared to a chymase that is not modified.
- [0218] Embodiment I-53. The engineered protease of any one of embodiments I-31 and I-52, wherein the engineered protease has an increased bioavailability compared to a chymase that is not modified.
- [0219] Embodiment I-54. The engineered protease of any one of embodiments I-1 to I-53, wherein the engineered protease is non-immunogenic.
- [0220] Embodiment I-55. The engineered protease of any one of embodiments Set I 1-54, wherein the engineered protease is in a zymogen form.
- [0221] Embodiment I-56. The engineered protease of any one of embodiments Set I 1-54, wherein the engineered protease is in an active form.
- [0222] Embodiment I-57. The engineered protease of any one of embodiments Set I 1-56, further comprising a half-life extender.
- [0223] Embodiment I-58. A method of inactivating Factor B, comprising contacting the Factor B with any of the engineered proteases of embodiments I-1 to I-57.
- [0224] Embodiment I-59. The method of Embodiment I-58, wherein complement activation is inhibited.
- [0225] Embodiment I-60. The method of Embodiment I-59, wherein the classical pathway of the complement pathway is inhibited.
- [0226] Embodiment I-61. The method of any one of embodiments 59-60, wherein the alternate pathway of the complement pathway is inhibited.

[0227] Embodiment I-62. The method of any one of embodiments 59-61, wherein the lectin pathway of the complement pathway is inhibited.

[0228] Embodiment I-63. The method of any one of embodiments set I 58-62, wherein the method is in vitro.

[0229] Embodiment I-64. The method of any one of embodiments Set I 58-62, wherein the method is in vivo.

[0230] Embodiment I-65. A method of treating a disease or condition in a subject in need thereof, comprising administering to the subject any one of the engineered proteases of embodiments 1-57.

[0231] Embodiment I-66. The method of Embodiment I-65, wherein the disease or condition is associated with dysregulated complement.

[0232] Embodiment I-67. The method of any one of embodiments Set I 65-66, wherein the disease or condition is an inflammatory disease or condition.

[0233] Embodiment I-68. The method of any one of embodiments 65-67, wherein the treatment is a replacement therapy.

[0234] Embodiment I-69. The method of any one of embodiments Set I 65-68, wherein the treatment blocks complement activation.

[0235] Embodiment I-70. The method of any one of embodiments Set I 65-69, wherein the treatment modulates autoimmunity.

[0236] Embodiment I-71. The method of any one of embodiments Set I 65-70, wherein the disease or condition is a congenital complement deficiency.

[0237] Embodiment I-72. The method of any one of embodiments Set I 65-71, wherein the treatment is for endothelial or kidney cell injury.

[0238] Embodiment I-73. The method of any one of embodiments Set I 65-72, wherein the disease or condition is selected from lupus nephritis, C3 glomerulopathy (C3G), primary IgA nephropathy, kidney transplant ischemia and reperfusion (I/R) injury, antineutrophil cytoplasmic

antibody (ANCA)-associated vasculitis (AAV), sepsis, acute respiratory distress syndrome (ARDS), SARS-associated coronavirus (SARS-CoV), atypical hemolytic uremic syndrome (aHUS), membranous nephropathy (MN) and paroxysmal nocturnal hemoglobinuria (PNH).

[0239] Embodiment I-74. The method of any one of embodiments Set I 65-73, wherein the disease or condition is a control protein deficiency.

[0240] Embodiment I-75. The method of any one of embodiments Set I 65-73, wherein the disease or condition is a secondary complement disorder.

[0241] Embodiment I-76. The method of any one of embodiments Set I 65-73, wherein the disease or condition is an immunity-related disease or condition.

[0242] Embodiment I-77. The method of any one of embodiments Set I 65-76, wherein the engineered protease is administered to the subject subcutaneously.

[0243] Embodiment I-78. The method of Embodiment I-77, wherein the engineered protease is activated in situ at the site of a dysregulated complement component.

[0244] Embodiment I-79. The method of any of any one of embodiments Set I 65-78, wherein the engineered protease is provided in a liquid stable formulation.

[0245] Embodiment I-80. A pharmaceutical composition comprising any of the engineered proteases of embodiments Set I 1-57, and optionally a pharmaceutically acceptable carrier.

[0246] Embodiment I-81. The pharmaceutical composition of Embodiment I-80, wherein the engineered protease is provided in a liquid stable formulation.

[0247] Embodiment I-82. The pharmaceutical composition of any one of embodiments Set I 80-81, wherein the composition is formulated for subcutaneous administration.

Set II

[0248] Embodiment II-1. An engineered protease comprising a modified chymase protease domain, a modified membrane type serine protease 1 (MTSP-1) protease domain, a modified urokinase-type plasminogen activator (uPA) protease domain, or a modified Kallikrein-related

peptidase 5 (KLK5) protease domain, wherein the engineered protease is capable of cleaving Factor B.

[0249] Embodiment II-2. The engineered protease of embodiment II-1, wherein cleavage of Factor B by the engineered protease generates one or more functionally inactive fragments.

[0250] Embodiment II-3. The engineered protease of embodiment II-2, wherein the one or more functionally inactive fragments are capable of reducing complement activation.

[0251] Embodiment II-4. The engineered protease of any one of embodiments II-1 to II-3, wherein cleavage of Factor B results in the generation of a Factor B fragment that is reduced in function.

[0252] Embodiment II-5. The engineered protease of any one of embodiments II-1 to II-4, wherein the Factor B is a non-human primate Factor B.

[0253] Embodiment II-6. The engineered protease of embodiment II-5, wherein the non-human primate is cynomolgus monkey.

[0254] Embodiment II-7. The engineered protease of any one of embodiments II-1 to II-4, wherein the Factor B is human Factor B.

[0255] Embodiment II-8. The engineered protease of embodiment II-7, wherein the Factor B comprises the amino acid sequence as set forth in SEQ ID NO: 1.

[0256] Embodiment II-9. The engineered protease of any one of embodiments II-1 to II-8, wherein cleavage of Factor B occurs at a site not targeted by Factor D.

[0257] Embodiment II-10. The engineered protease of embodiment II-9, wherein cleavage at the site not targeted by Factor D generates at least two fragments that are not Ba and Bb.

[0258] Embodiment II-11. The engineered protease of any one of embodiments II-1 to II-10, wherein cleavage of Factor B results in a reduction of the generation of Factor B cleavage products Ba and Bb as compared to cleavage by Factor D.

[0259] Embodiment II-12. The engineered protease of any one of embodiments II-1 to II-8, wherein cleavage of Factor B occurs at a site that is targeted by Factor D.

[0260] Embodiment II-13. The engineered protease of embodiment II-12, wherein the Factor B cleavage site targeted by Factor D comprises QQKR/KIV (SEQ ID NO: 9).

[0261] Embodiment II-14. The engineered protease of embodiment II-9, wherein the Factor B cleavage site comprises a sequence selected from: WEHR/KGT (SEQ ID NO: 10), KNQKR/QKQ (SEQ ID NO: 11), DVFY/QMI (SEQ ID NO: 12), EGVD/AE (SEQ ID NO: 13), DHKL/KSG (SEQ ID NO: 14), TPW/SLA (SEQ ID NO: 15), KVSEAD (SEQ ID NO: 20), IRPSKG (SEQ ID NO: 4), GGEKRD (SEQ ID NO: 5), GKKEAG (SEQ ID NO: 3), and DHKL/KSG (SEQ ID NO: 21).

[0262] Embodiment II-15. The engineered protease of embodiment II-9, wherein the Factor B cleavage site comprises a sequence selected from WEHR/KGT (SEQ ID NO: 10) and KNQKR/QKQ (SEQ ID NO: 11), and wherein the engineered protease comprises a modified MTSP-1 protease domain or a modified uPA protease domain.

[0263] Embodiment II-16. The engineered protease of any one of embodiments II-1 to II-15, wherein the engineered protease comprises a modified MTSP-1 protease domain.

[0264] Embodiment II-17. The engineered protease of any one of embodiments II-1 to II-15, wherein the engineered protease does not comprise a modified MTSP-1 protease domain.

[0265] Embodiment II-18. The engineered protease of embodiment II-16, comprising one or more modifications with respect to a MTSP-1 protease domain comprising an amino acid sequence as set forth in SEQ ID NO: 7.

[0266] Embodiment II-19. The engineered protease of embodiment II-18, wherein the modification is one or more of a substitution, an addition, and deletion of one or more amino acid residues.

[0267] Embodiment II-20. The engineered protease of embodiment II-16, wherein the one or more modifications is at one or more positions corresponding to one or more positions selected from D622, I640, L678, A686, F703, D705, F706, T707, F708, K719, C731, D734, Y755, Q783, V791, Q802, A814, D828, and K835 in a MTSP-1 protease domain comprising the sequence of amino acids set forth in SEQ ID NO: 18.

[0268] Embodiment II-21. The engineered protease of embodiment II-16, wherein the one or more modifications are selected from those presented in Table 5A.

[0269] Embodiment II-22. The engineered protease of embodiment II-16, wherein the one or more modifications are selected from those exemplary mutation strings presented in Table 5B.

[0270] Embodiment II-23. The engineered protease of any one of embodiments II-1 to II-15, wherein the engineered protease comprises a modified uPA protease domain.

[0271] Embodiment II-24. The engineered protease of any one of embodiments II-1 to II-15, wherein the engineered protease does not comprise a modified uPA protease domain.

[0272] Embodiment II-25. The engineered protease of embodiment II-23, comprising one or more modifications with respect to a uPA protease domain comprising an amino acid sequence as set forth in SEQ ID NO: 8.

[0273] Embodiment II-26. The engineered protease of embodiment II-25, wherein the modification is one or more of a substitution, an addition, and deletion of one or more amino acid residues.

[0274] Embodiment II-27. The engineered protease of embodiment II-23, wherein the one or more modifications is at one or more positions corresponding to one or more positions selected from S37, D60, L97, G161, R179, H180, V185, Y187, I207, A247, D248, A251, H252, C279, Y308, V316, A343, Q353, R378, K385 in a uPA protease domain comprising the sequence of amino acids set forth SEQ ID NO: 8.

[0275] Embodiment II-28. The engineered protease of embodiment II-23, wherein the one or more modifications are selected from those presented in Table 3A.

[0276] Embodiment II-29. The engineered protease of embodiment II-23, wherein the one or more modifications are selected from those exemplary mutation strings presented in Table 3B.

[0277] Embodiment II-30. The engineered protease of any one of embodiments II-1 to II-19, wherein the engineered protease comprises a modified chymase protease domain.

[0278] Embodiment II-31. The engineered protease of any one of embodiments II-1 to II-19, wherein the engineered protease does not comprise a modified chymase protease domain.

[0279] Embodiment II-32. The engineered protease of embodiment II-30, wherein the engineered protease comprises a modified chymase protease domain, and the cleavage site comprises a sequence selected from DVFY/QMI (SEQ ID NO: 12), EGVD/AE (SEQ ID NO: 13), DHKL/KSG (SEQ ID NO: 14), and TPW/SLA (SEQ ID NO: 15).

[0280] Embodiment II-33. The engineered protease of embodiment II-30, comprising one or more modifications with respect to a chymase protease domain comprising an amino acid sequence as set forth in SEQ ID NO: 6.

[0281] Embodiment II-34. The engineered protease of embodiment II-33, wherein the modification is one or more of a substitution, an addition, and deletion of one or more amino acid residues.

[0282] Embodiment II-35. The engineered protease of embodiment II-30, wherein the one or more modifications is one or more positions corresponding to one or more positions selected from S36 C7, V21, P26, G31, R37, K74, K80, I90, L101, L103, F110, V125, F157, D159, S176, A177, F178, K179, L186, V196, G199, A203, A207, F209 in a chymase protease domain comprising the sequence of amino acids set forth in SEQ ID NO: 6.

[0283] Embodiment II-36. The engineered protease of embodiment II-30, wherein the one or more modifications are selected from those presented in Table 7A.

[0284] Embodiment II-37. The engineered protease of embodiment II-30, wherein the one or more modifications are selected from those exemplary mutation strings presented in Table 7B.

[0285] Embodiment II-38. The engineered protease of any one of embodiments II-1 to II-19, wherein the engineered protease comprises a modified KLK5 protease domain, optionally comprising one or more amino acid modifications of SEQ ID NO: 23.

[0286] Embodiment II-39. The engineered protease of any one of embodiments II-1 to II-19, wherein the engineered protease does not comprise a modified KLK5 protease domain.

[0287] Embodiment II-40. The engineered protease of any one of embodiments II-1 to II-39, wherein the engineered protease has a k_{cat}/K_m of about 100, about 200, about 300, about 400, about 500, about 600, about 700, about 800, about 900, about 1,000, about 1,000, about 1,100, about 1,200, about 1,300, about 1,400, about 1,500, about 1,600, about 1,700, about 1,800, or about $1,900 \text{ M}^{-1} \text{ s}^{-1}$ for Factor B cleavage.

[0288] Embodiment II-41. The engineered protease of any one of embodiments II-1 to II-40, wherein the engineered protease has a k_{cat}/K_m of about 10^3 to about $10^9 \text{ M}^{-1} \text{ s}^{-1}$ for Factor B cleavage.

[0289] Embodiment II-42. The engineered protease of any one of embodiments II-1 to II-41, wherein the engineered protease has an EC_{50} for Factor B of less than about 20 nM.

[0290] Embodiment II-43. The engineered protease of any one of embodiments II-1 to II-42, wherein the engineered protease has an EC_{50} for Factor B of less than about 1 nM.

[0291] Embodiment II-44. The engineered protease of any one of embodiments II-1 to II-41, wherein the engineered protease has an EC_{50} for Factor B of about 20, about 25, or about 60 nM.

[0292] Embodiment II-45. The engineered protease of any one of embodiments II-1 to II-41, wherein the engineered protease has an EC_{50} for cleaving Factor B of about 1,000 to about 4,500 nM.

[0293] Embodiment II-46. The engineered protease of any one of embodiments II-1 to II-45, wherein the engineered protease has a plasma half-life in human plasma of over about 72 hours.

[0294] Embodiment II-47. The engineered protease of any one of embodiments II-1 to II-46, wherein the engineered protease has a plasma half-life in human plasma of over about 120 hours.

[0295] Embodiment II-48. The engineered protease of any one of embodiments II-1 to II-47, wherein the engineered protease has a plasma half-life in human plasma of about 7 days.

[0296] Embodiment II-49. The engineered protease of embodiment II-48, wherein the catalytic activity is about 10% to about 50%, or about 90% to about 100%.

[0297] Embodiment II-50. The engineered protease of embodiment II-16, wherein the engineered protease has an increased half-life compared to protease comprising a MTSP-1 protease domain that is not modified.

[0298] Embodiment II-51. The engineered protease of embodiment II-16, wherein the engineered protease has an increased bioavailability compared to an protease comprising a MTSP-1 protease domain that is not modified.

[0299] Embodiment II-52. The engineered protease of embodiment II-23, wherein the engineered protease has an increased half-life compared to a protease comprising a uPA protease domain that is not modified.

[0300] Embodiment II-53. The engineered protease of embodiment II-23, wherein the engineered protease has an increased bioavailability compared to a protease comprising a uPA protease domain that is not modified.

[0301] Embodiment II-54. The engineered protease of embodiment II-30, wherein the engineered protease has an increased half-life compared to protease comprising a chymase protease domain that is not modified.

[0302] Embodiment II-55. The engineered protease of embodiment II-30, wherein the engineered protease has an increased bioavailability compared to protease comprising a chymase protease domain that is not modified.

[0303] Embodiment II-56. The engineered protease of any one of embodiments II-1 to II-55, wherein the engineered protease is non-immunogenic.

[0304] Embodiment II-57. The engineered protease of any one of embodiments II-1 to II-56, wherein the engineered protease is in a zymogen form.

[0305] Embodiment II-58. The engineered protease of any one of embodiments II-1 to II-56, wherein the engineered protease is in an active form.

[0306] Embodiment II-59. The engineered protease of any one of embodiments II-1 to II-58, wherein the engineered protease is fused to a component that extends the half-life of the engineered protease.

[0307] Embodiment II-60. The engineered protease of embodiment II-59, wherein the component is a Fc domain.

[0308] Embodiment II-61. The engineered protease of embodiment II-59, wherein the component is a human serum albumin.

[0309] Embodiment II-62. The engineered protease of any one of embodiments II-1 to II-15, comprising a modified chymase protease domain having at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 95% sequence identity to SEQ ID NO: 6.

[0310] Embodiment II-63. The engineered protease of embodiment II-62, wherein the modified chymase protease domain of SEQ ID NO: 6 comprises one of the mutation strings of Table 7B.

[0311] Embodiment II-64. The engineered protease of any one of embodiments II-1 to II-15, comprising a modified membrane type serine protease 1 (MTSP-1) protease domain having at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 95% sequence identity to SEQ ID NO: 7.

[0312] Embodiment II-65. The engineered protease of embodiment II-64, wherein the modified MTSP-1 protease domain of SEQ ID NO: 7 comprises one of the mutation strings of Table 5B.

[0313] Embodiment II-66. The engineered protease of any one of embodiments II-1 to II-15, comprising a modified urokinase-type plasminogen activator (uPA) protease domain having at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 95% sequence identity to SEQ ID NO: 22.

[0314] Embodiment II-67. The engineered protease of embodiment II-66, wherein the modified uPA protease domain of SEQ ID NO: 22 comprises one of the mutation strings of Table 3B.

[0315] Embodiment II-68. The engineered protease of embodiment II-1, comprising a modified Kallikrein-related peptidase 5 (KLK5) protease domain having at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 95% sequence identity to SEQ ID NO: 23.

[0316] Embodiment II-69. A method of inactivating Factor B, comprising contacting the Factor B with any of the engineered proteases of embodiments II-1 to II-68.

[0317] Embodiment II-70. A method of treating a disease or condition in a subject in need thereof, comprising administering to the subject any one of the engineered proteases of embodiments II-1 to II-68.

[0318] Embodiment II-71. The method of embodiment II-70, wherein the disease or condition is associated with dysregulated complement.

[0319] Embodiment II-72. The method of any one of embodiments II-70 to II-71, wherein the disease or condition is an inflammatory disease or condition.

[0320] Embodiment II-73. The method of any one of embodiments II-70 to II-72, wherein the treatment is a replacement therapy.

[0321] Embodiment II-74. The method of any one of embodiments II-70 to II-73, wherein the treatment blocks complement activation.

[0322] Embodiment II-75. The method of any one of embodiments II-70 to II-74, wherein the treatment modulates autoimmunity.

[0323] Embodiment II-76. The method of any one of embodiments II-70 to II-75, wherein the disease or condition is a congenital complement deficiency.

[0324] Embodiment II-77. The method of any one of embodiments II-70 to II-76, wherein the treatment is for endothelial or kidney cell injury.

[0325] Embodiment II-78. The method of any one of embodiments II-70 to II-77, wherein the disease or condition is selected from lupus nephritis, C3 glomerulopathy (C3G), primary IgA nephropathy, kidney transplant ischemia and reperfusion (I/R) injury, antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV), sepsis, acute respiratory distress syndrome (ARDS), SARS-associated coronavirus (SARS-CoV), atypical hemolytic uremic syndrome (aHUS), membranous nephropathy (MN) and paroxysmal nocturnal hemoglobinuria (PNH).

[0326] Embodiment II-79. The method of any one of embodiments II-70 to II-78, wherein the disease or condition is a control protein deficiency.

[0327] Embodiment II-80. The method of any one of embodiments II-70 to II-78, wherein the disease or condition is a secondary complement disorder.

[0328] Embodiment II-81. The method of any one of embodiments II-70 to II-78, wherein the disease or condition is an immunity-related disease or condition.

[0329] Embodiment II-82. The method of any one of embodiments II-70 to II-81, wherein the engineered protease is administered to the subject subcutaneously.

[0330] Embodiment II-83. The method of embodiment II-82, wherein the engineered protease is activated in situ at the site of a dysregulated complement component.

[0331] Embodiment II-84. The method of any of any one of embodiments II-70 to II-83, wherein the engineered protease is provided in a liquid stable formulation.

[0332] Embodiment II-85. A pharmaceutical composition comprising any of the engineered proteases of embodiments II-1 to II-68, and optionally a pharmaceutically acceptable carrier.

[0333] Embodiment II-86. The pharmaceutical composition of embodiment II-85, wherein the engineered protease is provided in a liquid stable formulation.

[0334] Embodiment II-87. The pharmaceutical composition of any one of embodiments II-85 to II-86, wherein the composition is formulated for subcutaneous administration.

EXAMPLES

Example 1: Expression and Purification of Chymotrypsin-Like Serine Proteases and Initial Characterization

Expression and purification of MTSP-1- and uPA-Based Proteases

[0335] Small-scale expression and purification of engineered MTSP-1- and uPA-based proteases was performed. Briefly, MTSP-1 and uPA each were expressed as a zymogen in an *E. coli* strain of BL21-Gold (DE3) bacterial host, isolated from inclusion bodies, denatured, refolded by rapid dilution, dialyzed and subsequently activated on an immobilized trypsin column. The active protein was then purified on an anion exchange column. Similarly, large-scale refold and purification of MTSP-1 was also performed. The MTSP-1 and uPA were solubilized, refolded, purified on an anion exchange column. Next, endotoxin was removed from the MTSP-1 and uPA.

Expression and Purification of Chymase-Based Proteases

[0336] Small-scale expression and purification of chymase was performed. Briefly, chymase was expressed as a zymogen in inclusion bodies in an *E. coli* strain of BL21 Gold (DE3) bacterial host. The insoluble chymase was isolated from inclusion bodies, denatured in the presence of reducing agent, refolded by rapid dilution, and dialyzed. The zymogen form of the protein was purified using 4 mL of Fast Flow SP beads packed in BioRAD columns using gravity and activated overnight with enterokinase. The native chymase was then purified from the zymogen using cation exchange chromatography using a step elution method.

Factor B Cleavage by Chymase-Based Engineered Proteases

[0337] Factor B cleavage by chymase-based engineered proteases was tested and verified by Coomassie gel. Briefly, a digestion reaction was prepared with 2.0 μ M of Human Factor B (Complement Technologies) in 20 μ L buffer (50 mM Tris pH 7.4/50 mM NaCl/0.01% Tween 20). Different concentration of the chymase-based engineered proteases were added to the Factor B (3000 nM at the top, diluted 1:2, 10 steps, including 0.0nM) and were incubated for 1 hour at

37°C. After digestion, 15 µL of the reaction was transferred to a 96 well plate with 1.5 µL of 0.2N HCL to stop the chymase digestion. After quenching, the reactions were prepared for SDS-PAGE. 20 µL of the reaction mixtures were loaded per well of a 4-12% Bis-Tris Criterion gel. Densitometry analysis of the Factor B cleavage was performed, and EC₅₀ was calculated. FIG. 1D depicts a Coomassie gel showing examples of Factor B cleavage by the tested chymase-based engineered proteases. Table 8A lists the chymase-based engineered proteases tested in Fig. 1D. All references to engineered proteases in the table are depicted in chymotrypsin numbering. See Table 6 for the chymotrypsin numbering key for the modified protease domain of chymase. Two lots of each engineered protease set were used, and each lot showed the ability of the engineered proteases to cleave Factor B. Lot 1 of each set showed a visible amount of zymogen which is not titratable, but possibly is activated or is somewhat active on Factor B. These results also show that the engineered protease is highly efficient at Factor B cleavage. The engineered proteases tested are shown in Table 8A below.

[0338] FIG. 1E depicts a graph showing examples of Factor B cleavage by two chymase-based engineered proteases having low EC₅₀ calculated from ELISA measurements. All references in this Example to engineered proteases are depicted in chymotrypsin numbering. See Table 6 for the chymotrypsin numbering key for the modified protease domain of chymase. Briefly, a digestion reaction was prepared with 2.0 µM of Human Factor B (Complement Technologies) in 20 µL buffer (50 mM Tris pH 7.4/50 mM NaCl/0.01% Tween 20). Different concentrations of two chymase-based engineered proteases were used: C22S/P38Q/K40L/F41R/V138I/F173Y/D175N/A190S/V213A/S218V/A226R and C22S/P38Q/K40M/F41R/V138I/F173Y/D175R/A190S/V213A/S218V/A226R. These were each provided at 3000 nM, and diluted 1:2 to obtain 1500, 750, 375, 188, 94, 47, 23, 12, 6, and 3 nM, and additionally a control at 0.0nM was used. These were each added to 2 µM of Factor B and incubated for 1 hour at 37°C. After digestion, 15 µL of the reaction was transferred to a 96 well plate with 1.5 µL of 0.2N HCL. After quenching, the reactions were prepared Factor B ELISA.

[0339] A Factor B standard curve was made with 800, 533.3, 355.5, 237.0, 158.0, 105.3, 70.2, 46.8, 31.2, 20.8, 0.0 pM in 1% BSA-PBST. A 384 well plate was coated with the monoclonal antibody against the human Factor Ba fragment (Quidel) at 2 µg/ml in carbonate buffer (25 µL/well). After blocking for 1 hour at room temperature with 1% BSA-PBST (100 µL/well), the

digests (chymase + FB) were diluted to 800 pM and the standards were diluted 1:1.5 from 800 pM into the blocking buffer (25 μ L/well). The plate was agitated for 30 minutes at room temperature.

[0340] The biotinylated monoclonal antibody against the human Factor Bb fragment (Quidel) was then added at 0.125 μ g/ml to detect the bound FB. Streptavidin-HRP was then diluted at 1:200 in blocking buffer (25 μ L/well) and the plate was agitated for 30 minutes at room temperature. The plate was developed with ELISABright, (50 μ L/well) for 1 minute at room temperature and read in the EnVision plate reader. Table 8B below presents the EC₅₀ calculated for the two engineered proteases. FIG. 1E shows that 30-42 nM of the chymase-based engineered proteases tested were required to inhibit 50% of Factor B.

Table 8A: Chymase-Based Engineered Proteases Tested in FIG. 1D

Notation in FIG. 1D	Mutation of Engineered Protease
1	C22S/K40L/F41K/L99N/F173Y/D175N/S218L/A226R
2	C22S/P38Q/K40M/F41R/L99M/F173Y/D175N/V213Q/S218V/A226R/F228A
3	C22S/P38Q/K40M/F41R/D175N/V213Q/S218V/A226R/F228A
4	C22S/P38Q/K40L/F41R/L99H/V138I/F173Y/D175S/A190S/V213A/S218I/A226R

Table 8B: EC₅₀ of Factor B Cleavage by Chymase-Based Engineered Proteases

	C22S/P38Q/K40L/F41R/V138I/F173Y/D175N/A190S/V213A/S218V/A226R	C22S/P38Q/K40M/F41R/V138I/F173Y/D175R/A190S/V213A/S218V/A226R
EC ₅₀	42.47	30.22

Example 2: Protein Characterization and Active Site Titration of Chymase-Based Engineered Proteases

[0341] Selected chymase-based engineered proteases were tested to measure the activity of these proteases, by using an active site titration method based on reaction with the inhibitory serpin bait, followed by HPLC analysis to quantitate the active fraction. All references in this Example, including Table 9, to engineered proteases are depicted in chymotrypsin numbering. See Table 6 for the chymotrypsin numbering key for the modified protease domain of chymase. The HPLC analysis detects a shift in the measured peak when the serpin bait is present, an indication that the protease is binding the bait and therefore active. Briefly, a working concentration of 24 μM of serpin bait was prepared from stock solution, with 50 μM of low molecular weight heparin for antithrombin (AT)-based baits. Protease solutions were prepared by using 30 μL of a 5 μM working solution. Reaction mixtures were incubated for 2 hours at 37°C, and each reaction mixture (protease alone or protease with bait) was analyzed by HPLC using standard protocols. All protease alone samples were run first, then followed by the protease with serpin bait samples. A summary of the results for the proteases tested is summarized in Table 9 below. $k_{\text{cat}}/K_{\text{m}}$ was measured using EGVD/AE-QF (SEQ ID NO: 52) and second order rate constant K^*_{app} was measured using EGVD/AE-ACT (SEQ ID NO: 53). These results measured the protease activity after purification, and most produced chymase-based engineered proteases tested showed an activity of between 60%-120%.

Table 9: Active Site Titration of Chymase-Based Engineered Proteases Summary

Mutation String	k_{cat}/Km EGVD/AE-QF (SEQ ID NO: 52)	k*_{app} EGVD/AE- ACT (SEQ ID NO: 53)
C22S		30.2
C22S/A226R	347	77
C22S/L99H/F173L/K192R/Y215H/R217T/S218V/A226R	86	341
C22S/L99H/F173I/K192R/Y215K/R217T/S218I/A226R	202	1041
C22S/L99H/F173L/K192R/Y215H/R217T/S218V/A226R	374	2412
C22S/F41I/L99W/G151L/F173K/R217S/A226R	380	1732
C22S/F41R/L99G/G151T/F173Y/Y215R/R217M/A226R	385	773
C22S/F41R/L99H/G151T/F173Y/Y215K/R217M/A226R	197	1495
C22S/F41R/L99Y/G151T/F173K/R217S/A226R	68	684
C22S/F41V/L99F/G151R/F173K/R217S/A226R	102	652
C22S/F41V/L99H/G151R/F173L/Y215R/R217T/A226R	363	1131
F41V/L99H/G151V/F173Y/Y215K/R217G/A226R	457	649
C22S/F41V/L99W/G151L/F173K/R217S/A226R	413	1359
C22S/K192R/A226R	243	147
C22S/L99R/F173L/K192R/Y215H/R217T/S218V/A226R	229	
C22S/L99Y/F173L/K192R/Y215H/R217T/S218V/A226R	224	
C22S/L99H/F173Y/K192R/S218L/A226R	449	
C22S/L99Y/F173Y/K192R/S218L/A226R	231	
C22S/K40L/F41A/K192R/A226R	60	
C22S/K40M/F41V/K192R/A226R	32	
C22S/K40M/F41V/G151R/K192R/A226R	31	
C22S/L99H/F173Y/K192R/Y215K/R217S/S218V/A226R	262	
C22S/L99F/F173K/K192R/R217S/S218I/A226R	86	
C22S/L99W/F173K/K192R/R217S/S218V/A226R	128	
C22S/L99W/F173K/R217S/S218R/A226R	810	
C22S/L99Q/F173L/Y215K/R217T/S218L/A226R	55	
C22S/F41R/L99H/G151S/F173Y/Y215K/R217M/A226R	71	
C22S/F41I/L99H/G151K/F173Y/Y215K/R217G/A226R	89	
C22S/F41V/L99H/G151R/F173S/Y215K/A226R	53	

Mutation String	k _{cat} /K _m EGVD/AE-QF (SEQ ID NO: 52)	k [*] _{app} EGVD/AE- ACT (SEQ ID NO: 53)
C22S/F41R/L99H/F173Y/Y215K/R217M/A226R	30	
C22S/F41K/L99H/G151A/F173Y/Y215K/R217S/A226R	355	
C22S/F41R/L99H/G151A/F173S/Y215K/R217T/A226R	52	
C22S/F173Y/D175N/A226R	2687	
C22S/K40L/F41R/L99N/F173Y/S218L/A226R	197	
C22S/K40L/F41H/L99N/F173Y/S218L/A226R	190	
C22S/K40L/F41K/L99N/F173Y/S218L/A226R	194	
C22S/K40L/F41R/L99N/F173Y/D175N/S218L/A226R	793	
C22S/K40L/F41H/L99N/F173Y/D175N/S218L/A226R	638	
C22S/K40L/F41K/L99N/F173Y/D175N/S218L/A226R	721	
C22S/K40M/F41R/L99Q/F173Y/D175R/S218V/A226R	157	
C22S/K40M/F41R/L99Q/F173Y/D175R/S218I/A226R	241	
C22S/P38Q/V138I/A190S/V213A/A226R	241	
C22S/P38Q/V213Q/A226R/F228A	1030	
C22S/P38Q/K40A/F41R/L99H/D175N/V213Q/S218I/A226R/F228A	180	
C22S/P38Q/K40M/F41R/L99M/F173Y/D175N/V213Q/S218V/A226R /F228A	181	
C22S/P38Q/K40M/F41R/D175N/V213Q/S218V/A226R/F228A	198	
C22S/P38Q/K40L/F41R/V138I/F173Y/D175N/A190S/V213A/S218V/ A226R	1389	
C22S/P38Q/K40L/F41R/L99H/V138I/F173Y/D175S/A190S/V213A/S 218I/A226R	832	
C22S/P38Q/K40M/F41R/L99F/V138I/F173S/D175H/A190S/V213A/S 218V/A226R	95	
C22S/P38Q/K40A/F41R/L99H/V138I/F173Y/D175N/A190S/V213A/S 218V/A226R	863	
C22S/P38Q/K40A/F41K/L99H/V138I/D175S/A190S/V213A/S218V/ A226R	608	
C22S/P38Q/K40M/F41R/L99N/V138I/F173Y/D175T/A190S/V213A/ S218V/A226R	398	

Mutation String	k _{cat} /K _m EGVD/AE-QF (SEQ ID NO: 52)	k _{app} EGVD/AE- ACT (SEQ ID NO: 53)
C22S/P38Q/K40M/F41R/V138I/F173Y/D175R/A190S/V213A/S218V/A226R	1797	
C22S/P38Q/K40L/F41R/L99N/V138I/F173Y/D175N/A190S/V213A/S218V/A226R	420	

Example 3: Stability Studies of Serine Proteases Using Factor B Cleavage and Selection of Serine Proteases

[0342] Cleavage of Factor B by various engineered proteases was evaluated. Engineered chymase-, MTSP-1-, and uPA-based proteases were tested. All references in this Example, including Tables 10A, 10B, 11A, and 11B, to engineered proteases are depicted in chymotrypsin numbering. The chymotrypsin numbering key for the modified protease domains of engineered proteases are found in Tables 2, 4, and 6 for uPA, MTSP-1, and chymase, respectively. These experiments were also performed to evaluate the stability of non-naturally occurring chymases in various media, including cynomolgus monkey vitreous humor, human plasma, and phosphate buffered saline (PBS). Briefly, Factor B was diluted into an assay buffer and incubated at 37°C with naturally occurring (wild type) and non-naturally occurring (engineered) proteases in vitreous humor, plasma, or PBS, and the reactions were quenched with HCl at various time points. The time points tested for chymase include T = 0 which used 6 hours pre-incubation, T = 2 hours which used 4 hours pre-incubation, T = 4 hours which used 2 hours pre-incubation, and T = 6 hours which used 0 hours pre-incubation. Assaying of the sample was performed with ELISA, or alpha screen for human C3 cleavage, using standard protocols.

[0343] A summary of the results is presented in Tables 10A-10B below, showing the various tested proteases, the concentration of the protease used in nM and the EC₅₀ of human Factor B cleavage. Table 10 presents human Factor B cleavage by various non-naturally occurring chymase-based engineered proteases, tested in 80% human plasma, compared against a chymase comprising the mutation string C122S. Blank cells indicate that a test was not performed for a particular protease mutation string. These results generally show that selected chymase-based engineered proteases such as

C22S/P38Q/K40L/F41R/V138I/F173Y/D175N/A190S/V213A/S218V/A226R and C22S/P38Q/K40M/F41R/V138I/F173Y/D175R/A190S/V213A/S218V/A226R show high Factor B cleavage activity.

[0344] Table 10B presents human Factor B cleavage by various engineered chymase-, MTSP-1-, or uPA-based proteases, tested in mouse or human plasma. For ELISA analysis of Factor B cleavage, briefly, anti-Ba was used, and Bb was detected with anti-Bb. A dynamic range of 25-1600 pM was used. Half-life in 50% human plasma was also calculated for various chymase-based engineered proteases.

[0345] These results showed that the proteases engineered from the uPA-based scaffold did not show efficient Factor B cleavage, and proteases engineered from the MTSP-1-based scaffold showed Factor B cleavage but only for two candidates below 50 nM. The proteases engineered from the chymase-based scaffold showed the most efficient Factor B cleavage with EC₅₀ values below 50 nM.

Table 10A: Chymase-Based Engineered Proteases EC₅₀ For Cleavage of Factor B

Mutation String	fB Cleavage EC ₅₀ (nM)	fB Cleavage, 80% plasma EC ₅₀ (nM)	fB Cleavage, 64% plasma EC ₅₀ (nM)	fB Cleavage, 64% A ₂ M-depl plasma EC ₅₀ (nM)	t _{1/2} in 50% human plasma (min) (EGVD'A E)(SEQ ID NO: 13)
C22S	265	10200	7885	1519	
C22S/A226R	5205	none			
C22S/L99H/F173L/K192R/Y215H/R217T/S218V/A226R	7770				
C22S/L99H/F173I/K192R/Y215K/R217T/S218I/A226R	5618				
C22S/L99H/F173L/K192R/Y215H/R217T/S218V/A226R	2738				
C22S/F41I/L99W/G151L/F173K/R217S/A226R	1741				
C22S/F41R/L99G/G151T/F173Y/Y215R/R217M/A226R	4329				
C22S/F41R/L99H/G151T/F173Y/Y215K/R217M/A226R	1690				
C22S/F41R/L99Y/G151T/F173K/R217S/A226R	3447				

Mutation String	fB Cleavage EC ₅₀ (nM)	fB Cleavage, 80% plasma EC ₅₀ (nM)	fB Cleavage , 64% plasma EC ₅₀ (nM)	fB Cleavage, 64% A ₂ M- depl plasma EC ₅₀ (nM)	t _{1/2} in 50% human plasma (min) (EGVD'A E)(SEQ ID NO: 13)
C22S/F41V/L99F/G151R/F173 K/R217S/A226R	3646				
C22S/F41V/L99H/G151R/F173 L/Y215R/R217T/A226R	8060				
F41V/L99H/G151V/F173Y/Y21 5K/R217G/A226R	7770				
C22S/F41V/L99W/G151L/F173 K/R217S/A226R	1944				
C22S/K192R/A226R	6367				17.3
C22S/L99R/F173L/K192R/Y21 5H/R217T/S218V/A226R	7770	7590*	4380	2290	20.9
C22S/L99Y/F173L/K192R/Y21 5H/R217T/S218V/A226R	4880-6304		12406	4575	10.1
C22S/L99H/F173Y/K192R/S21 8L/A226R	1147				
C22S/L99R/F173Y/K192R/S21 8L/A226R	555				
C22S/L99Y/F173Y/K192R/S21 8L/A226R	1020				
C22S/K40L/F41A/K192R/A226 R	7390				
C22S/K40L/F41G/K192R/A226 R	1020 - 8540				
C22S/K40M/F41V/K192R/A22 6R	7770				
C22S/K40M/F41V/G151R/K19 2R/A226R	7770				
C22S/K40L/F41V/G151R/K192 R/A226R	7500				
C22S/L99H/F173Y/K192R/Y21 5K/R217S/S218V/A226R	3456				
C22S/L99F/F173K/K192R/R21 7S/S218I/A226R	4001				
C22S/L99W/F173K/K192R/R21 7S/S218V/A226R	2492				
C22S/L99W/F173K/R217S/S21 8R/A226R	2663				
C22S/L99Q/F173L/Y215K/R21 7T/S218L/A226R	(weak)				
C22S/F41R/L99H/G151S/F173 Y/Y215K/R217M/A226R	3387 - 2730				
C22S/F41I/L99H/G151K/F173Y /Y215K/R217G/A226R	7770				

Mutation String	fB Cleavage EC ₅₀ (nM)	fB Cleavage, 80% plasma EC ₅₀ (nM)	fB Cleavage , 64% plasma EC ₅₀ (nM)	fB Cleavage, 64% A ₂ M- depl plasma EC ₅₀ (nM)	t _{1/2} in 50% human plasma (min) (EGVD'A E)(SEQ ID NO: 13)
C22S/F41V/L99H/G151R/F173 S/Y215K/A226R	4781				
C22S/F41R/L99H/F173Y/Y215 K/R217M/A226R	6518 - 5390				
C22S/F41K/L99H/G151A/F173 Y/Y215K/R217S/A226R	1313	18600	5692	5752	
C22S/F41R/L99H/G151A/F173 S/Y215K/R217T/A226R	2250		3402	2879	
C22S/F41L/L99H/G151T/F173 Y/Y215K/A226R	3230				
C22S/F41R/L99H/G151T/F173 Y/Y215K/A226R	3220				
C22S/F41G/L99H/G151T/F173 Y/Y215K/A226R	6600				
C22S/F41R/L99H/F173Y/Y215 K/A226R	4800				
C22S/F41V/L99H/F173Y/Y215 K/A226R	6060				
C22S/F41G/L99H/F173Y/Y215 K/A226R	6650				
C22S/F41R/L99H/G151T/F173 L/Y215K/A226R	4800				
C22S/F41V/L99H/G151T/F173 L/Y215K/A226R	5420				
C22S/F41G/L99H/G151T/F173 L/Y215K/A226R	11500				
C22S/F41R/L99H/F173L/Y215 K/A226R	6640				
C22S/F41V/L99H/G151T/F173 Y/Y215K/A226R	5330				
C22S/F41L/L99H/F173Y/Y215 K/A226R	3450				
C22S/F41L/L99H/G151T/F173 L/Y215K/A226R	4170				
C22S/F41L/L99H/F173L/Y215 K/A226R	7890				
C22S/F41V/L99H/F173L/Y215 K/A226R	8730				
C22S/F41G/L99H/F173L/Y215 K/A226R	10200				
C22S/L99R/K192R/S218L/A22 6R	7510				
C22S/L99R/F173Y/S218L/A226 R	546				11.1

Mutation String	fB Cleavage EC ₅₀ (nM)	fB Cleavage, 80% plasma EC ₅₀ (nM)	fB Cleavage , 64% plasma EC ₅₀ (nM)	fB Cleavage, 64% A ₂ M- depl plasma EC ₅₀ (nM)	t _{1/2} in 50% human plasma (min) (EGVD'A E)(SEQ ID NO: 13)
C22S/L99R/F173Y/K192R/A226R	3278				
C22S/K93Q/G119D/F173S/K192R/A226R	7770				
C22S/P38Q/H100P/L116R/F123L/Q180H/S218I/A226R/R235S	868 - 1120				29
E21G/C22S/R61H/M135T/F173Y/D175N/A209V/A226R	1275 - 1050				23
C22S/K111E/F173L/Y215H/A226R	9008				
C22S/F173L/Y215H/A226R	5991				
C22S/M135T/P150S/S152G/Q166H/D175N/A190T/A226R	3163				
E21G/C22S/R61H/M135T/F173Y/D175N/A209V/A226R	1260				
C22S/P38Q/H100P/L116R/F123L/Q180H/A226R/R235S	3737				
C22S/V138I/L160V/S189T/A226R	7132				
C22S/V138L/A226R	3483				
C22S/V138L/A226R	3483				
C22S/L160V/S189T/A226R	6636				
C22S/S189T/A226R	7984				
C22S/S218I/A226R	830				7.8
C22S/F173Y/A226R	650	21000			5.9
C22S/D175N/A226R	564	7770			6.2
C22S/F173Y/D175N/A226R	151- 182 - 177	11200	5999 – 5616	1647	7.3, 9.0
C22S/A220S/A226R	5563				
C22S/S189T/A190S/F191S/A220L/A226R	8141				
C22S/K40L/F41R/L99N/F173Y/S218L/A226R	379	15568			22.7
C22S/K40M/F41R/L99H/G151P/F173S/S218V/A226R	1503 - 1910				
C22S/K40M/F41R/L99H/G151A/F173S/S218V/A226R	3221 - 4710				
C22S/K40M/F41I/L99H/G151R/F173S/S218L/A226R	8202				
C22S/K40M/F41R/L99N/G151S/F173Y/S218L/A226R	320 - 445	11193			10.9
C22S/K40M/F41R/L99N/G151P/F173S/S218V/A226R	2575 - 3010				

Mutation String	fB Cleavage EC ₅₀ (nM)	fB Cleavage, 80% plasma EC ₅₀ (nM)	fB Cleavage , 64% plasma EC ₅₀ (nM)	fB Cleavage, 64% A ₂ M- depl plasma EC ₅₀ (nM)	t _{1/2} in 50% human plasma (min) (EGVD'A E)(SEQ ID NO: 13)
C22S/K40M/F41R/L99H/G151P /F173S/S218I/A226R	1864 - 2100				
C22S/K40L/F41H/L99N/F173Y /S218L/A226R	284 - 530	13903			28.9
C22S/K40M/F41V/L99N/G151 R/F173S/S218V/A226R					
C22S/K40L/F41L/L99N/G151H /F173Y/S218V/A226R	304 - 746	17076			20.4
C22S/K40L/F41K/L99H/G151S /F173Y/S218L/A226R	276 - 369	17234			20.1
C22S/K40L/F41K/L99H/F173Y /S218L/A226R	228 - 394	14813			13.9
C22S/K40L/F41K/L99N/F173Y /S218L/A226R	405 - 373	13979			28.9
C22S/K40M/F41V/L99N/G151 R/F173S/S218E/A226R	11824				
C22S/A190S/A226R	407				4.2
C22S/S189T/A190S/A226R	605	none			
C22S/K40L/F41R/L99N/F173Y/ D175N/S218L/A226R	119	4092; 3756			23.7
C22S/K40L/F41H/L99N/F173Y /D175N/S218L/A226R	139	5066			24.9
C22S/K40L/F41K/L99N/F173Y /D175N/S218L/A226R	61 - 129	5697			27
C22S/P38Q/V138I/V183I/L199 V/A226R/F228V	none				
C22S/V138T/A190S/V213A/A2 26R	6708				
C22S/P38Q/V138L/A190G/L19 9V/V213A/A226R/F228A	none				
C22S/P38Q/V138I/A226R/F228 A	12395				
C22S/F41K/L99N/F173Y/D175 N/S218L/A226R	77				30
C22S/K40L/L99N/F173Y/D175 N/S218L/A226R	113				11.4
C22S/K40L/F41K/F173Y/D175 N/S218L/A226R	77				10.3
C22S/K40L/F41K/L99N/D175N /S218L/A226R	295				13.8
C22S/K40L/F41K/L99N/F173Y /D175N/A226R	151				15.1
C22S/V138A/G145D/S189T/A1 90S/A226R	3813				

Mutation String	fB Cleavage EC ₅₀ (nM)	fB Cleavage, 80% plasma EC ₅₀ (nM)	fB Cleavage , 64% plasma EC ₅₀ (nM)	fB Cleavage, 64% A ₂ M- depl plasma EC ₅₀ (nM)	t _{1/2} in 50% human plasma (min) (EGVD'A E)(SEQ ID NO: 13)
C22S/A226R/F228A	5072				
C22S/V138A/S189T/A226R/F228M	1772				
C22S/V138L/S189T/A190G/A226R	4160				
C22S/V138S/S189T/A226R/F228M	2985				
C22S/S189T/A226R/F228M	1553				
C22S/V138A/A226R	2942				
C22S/V138A/S189T/A226R	4420				
C22S/K40M/F41R/L99N/F173Y/D175S/S218L/A226R	230	5137			29.6
C22S/K40M/F41R/L99H/F173Y/D175W/S218L/A226R	181	7213			11.2
C22S/K40M/F41K/F173Y/D175R/S218E/A226R	203	4130			38.8
C22S/K40M/F41K/L99N/F173Y/D175R/S218T/A226R	216	4128			16.9
C22S/K40L/F41K/L99Q/F173Y/D175N/S218V/A226R	110	5672			15.6
C22S/K40M/F41R/L99Q/F173Y/D175R/S218V/A226R	235	4069			73.7
C22S/K40L/F41K/L99H/F173Y/D175S/S218I/A226R	101	4621			11.5
C22S/K40L/F41R/L99G/F173Y/D175R/S218V/A226R	333	8625			24.9
C22S/K40L/F41R/L99H/F173Y/D175Y/S218I/A226R	84	5561			10.4
C22S/K40M/F41R/L99Q/F173Y/D175R/S218I/A226R	213	4481			65.3
C22S/P38Q/V138I/A190S/V213A/A226R	1348.4				
C22S/P38Q/V213Q/A226R/F228A	6210.6				
C22S/P38Q/K40A/F41R/L99H/F173Y/D175Y/V213Q/S218V/A226R/F228A	768.8				
C22S/P38Q/K40A/F41R/L99H/D175N/V213Q/S218I/A226R/F228A	552.9				
C22S/P38Q/K40M/F41R/L99M/F173Y/D175N/V213Q/S218V/A226R/F228A	787.5				

Mutation String	fB Cleavage EC ₅₀ (nM)	fB Cleavage, 80% plasma EC ₅₀ (nM)	fB Cleavage , 64% plasma EC ₅₀ (nM)	fB Cleavage, 64% A ₂ M- depl plasma EC ₅₀ (nM)	t _{1/2} in 50% human plasma (min) (EGVD'A E)(SEQ ID NO: 13)
C22S/P38Q/K40M/F41R/D175 N/V213Q/S218V/A226R/F228A	931.5				
C22S/P38Q/K40L/F41R/V138I/ F173Y/D175N/A190S/V213A/S 218V/A226R	36.3				
C22S/P38Q/K40L/F41R/L99H/ V138I/F173Y/D175S/A190S/V2 13A/S218I/A226R	60.3				
C22S/P38Q/K40M/F41R/L99F/ V138I/F173S/D175H/A190S/V2 13A/S218V/A226R	625 - 796.6				
C22S/P38Q/K40A/F41R/L99H/ V138I/F173Y/D175N/A190S/V 213A/S218V/A226R	71.8				
C22S/P38Q/K40A/F41K/L99H/ V138I/D175S/A190S/V213A/S2 18V/A226R	142.3				
C22S/P38Q/K40M/F41R/L99N/ V138I/F173Y/D175T/A190S/V2 13A/S218V/A226R	92.1				
C22S/P38Q/K40M/F41R/V138I/ F173Y/D175R/A190S/V213A/S 218V/A226R	28.1				
C22S/P38Q/K40L/F41R/L99N/ V138I/F173Y/D175N/A190S/V 213A/S218V/A226R	65.3				
C22S/P38Q/K40A/F41R/V138I/ D175R/A190S/V213A/S218T/A 226R	261.7				
C22S/P38Q/K40A/F41R/L99H/ V138I/F173Y/A190S/V213A/S2 18V/A226R	405.5				
C22S/P38Q/K40M/F41H/L99N/ V138I/F173Y/D175S/A190S/V2 13A/S218T/A226R	561				
C22S/P38Q/K40M/F41K/L99Y/ V138I/F173M/D175Y/A190S/V 213A/S218V/A226R	448.1				
C22S/P38Q/K40A/F41R/V138I/ F173S/D175T/A190S/V213A/S 218V/A226R	1720.7				
C22S/P38Q/K40L/F41R/V138I/ F173L/D175H/A190S/V213A/S 218V/A226R	314.2				

Mutation String	fB Cleavage EC ₅₀ (nM)	fB Cleavage, 80% plasma EC ₅₀ (nM)	fB Cleavage , 64% plasma EC ₅₀ (nM)	fB Cleavage, 64% A ₂ M- depl plasma EC ₅₀ (nM)	t _{1/2} in 50% human plasma (min) (EGVD'A E)(SEQ ID NO: 13)
C22S/P38Q/K40M/F41R/L99N/ V138I/D175N/A190S/V213A/S 218T/A226R	448.9				
C22S/P38Q/K40M/F41H/V138I /F173Y/D175R/A190S/V213A/ S218V/A226R	47.6				
C22S/P38Q/K40M/F41R/L99Q/ V138I/F173Y/D175A/A190S/V 213A/S218T/A226R	723.6				
C22S/P38Q/K40A/F41R/L99H/ V138I/D175R/A190S/V213A/S 218L/A226R	190.7				
C22S/P38Q/K40M/F41R/L99F/ V138I/F173L/D175N/A190S/V2 13A/A226R	870.1				
C22S/P38Q/K40M/F41R/L99Y/ V138I/F173L/D175S/A190S/V2 13A/S218V/A226R	814.2				
C22S/P38Q/K40L/F41R/V138I/ D175N/A190S/V213A/S218V/A 226R	114.5				
C22S/P38Q/K40M/F41R/L99Y/ V138I/F173Y/D175S/A190S/V2 13A/S218V/A226R	102.7				
C22S/P38Q/K40L/F41R/V138I/ F173M/D175N/A190S/V213A/S 218L/A226R	456.4				
C22S/P38Q/K40L/F41R/L99N/ V138I/F173Y/D175T/A190S/V2 13A/S218V/A226R	57.6				
C22S/P38Q/K40M/F41R/V138I/ F173L/D175N/A190S/V213A/S 218L/A226R	481.7				
C22S/P38Q/K40L/F41K/L99N/ V138I/F173Y/D175S/A190S/V2 13A/S218I/A226R	143.5				
C22S/P38Q/K40A/F41R/L99Y/ V138I/F173L/D175R/A190S/V2 13A/S218L/A226R	510.7				
C22S/P38Q/K40L/F41R/L99Q/ V138I/F173Y/D175N/A190S/V 213A/S218V/A226R	102.5				
C22S/P38Q/K40M/F41R/L99Q/ V138I/F173Y/D175N/A190S/V 213A/S218V/A226R	157.3				

Mutation String	fB Cleavage EC ₅₀ (nM)	fB Cleavage, 80% plasma EC ₅₀ (nM)	fB Cleavage , 64% plasma EC ₅₀ (nM)	fB Cleavage, 64% A ₂ M- depl plasma EC ₅₀ (nM)	t _{1/2} in 50% human plasma (min) (EGVD'A E)(SEQ ID NO: 13)
C22S/P38Q/K40V/F41R/V138I/ F173L/D175R/A190S/V213A/S 218I/A226R	169				
C22S/P38Q/K40M/F41T/V138I/ D175R/A190S/V213A/S218I/A 226R	218.4				
C22S/P38Q/K40M/F41R/V138I/ F173L/D175K/A190S/V213A/S 218V/A226R	349.5				
C22S/P38Q/K40M/F41R/L99Y/ V138I/F173S/D175S/A190S/V2 13A/S218T/A226R	10564.6				
C22S/P38Q/K40A/F41T/L99Y/ V138I/F173S/D175H/A190S/V2 13A/S218I/A226R	3125.4				
C22S/P38Q/K40A/F41R/V138I/ F173L/D175R/A190S/V213A/S 218L/A226R	282.9				
C22S/P38Q/K40M/F41R/L99Y/ V138I/F173L/D175Q/A190S/V2 13A/S218T/A226R	1650.6				
C22S/P38Q/K40A/F41Q/V138I/ F173S/D175W/A190S/V213A/S 218V/A226R	2187.2				
C22S/P38Q/K40M/F41H/V138I /D175S/A190S/V213A/A226R	1060.4				
C22S/P38Q/K40V/F41R/V138I/ F173L/D175N/A190S/V213A/S 218V/A226R	279.1				
C22S/P38Q/K40V/F41R/L99H/ V138I/F173M/D175N/A190S/V 213A/S218T/A226R	638.9				
C22S/P38Q/K40L/F41R/V138I/ F173L/D175R/A190S/V213A/S 218V/A226R	112.9				
C22S/P38Q/K40L/F41R/L99M/ V138I/F173L/D175R/A190S/V2 13A/S218V/A226R	212.8				
C22S/P38Q/K40A/F41R/L99H/ V138I/F173M/D175T/A190S/V 213A/S218I/A226R	882				
C22S/P38Q/K40M/F41R/L99Y/ V138I/F173L/D175S/A190S/V2 13A/S218T/A226R	1225.5				

Mutation String	fB Cleavage EC ₅₀ (nM)	fB Cleavage, 80% plasma EC ₅₀ (nM)	fB Cleavage , 64% plasma EC ₅₀ (nM)	fB Cleavage, 64% A ₂ M- depl plasma EC ₅₀ (nM)	t _{1/2} in 50% human plasma (min) (EGVD'A E)(SEQ ID NO: 13)
C22S/P38Q/K40A/F41R/L99H/ V138I/F173Y/D175N/A190S/V 213A/S218I/A226R	92.9				
C22S/P38Q/K40M/F41R/L99W/ V138I/F173M/D175H/A190S/V 213A/S218T/A226R	414.5				
C22S/P38Q/K40M/F41H/L99H/ V138I/D175S/A190S/V213A/S2 18V/A226R	277.4				
C22S/P38Q/K40V/F41R/V138I/ F173S/D175N/A190S/V213A/S 218I/A226R	716.7				
C22S/P38Q/K40L/F41R/L99F/ V138I/F173L/D175N/A190S/V2 13A/A226R	631.2				
C22S/P38Q/K40M/F41R/L99H/ V138I/D175Q/A190S/V213A/S 218V/A226R	300.8				
C22S/P38Q/K40L/F41R/V138I/ F173Y/D175H/A190S/V213A/S 218T/A226R	121.2				
C22S/P38Q/K40M/F41K/L99W/ V138I/F173M/A190S/V213A/S 218N/A226R	956.5				
C22S/P38Q/K40Q/F41R/V138I/ F173Y/D175N/A190S/V213A/S 218V/A226R	100.2				
C22S/P38Q/K40I/F41R/L99N/V 138I/D175T/A190S/V213A/S21 8V/A226R	202.9				
C22S/P38Q/K40Q/F41R/L99H/ V138I/F173Y/D175S/A190S/V2 13A/S218V/A226R	149.9				
C22S/P38Q/K40A/F41R/L99H/ V138I/F173Y/D175N/A190S/V 213A/S218L/A226R	138.6				
C22S/P38Q/K40M/F41R/L99N/ V138I/F173Y/D175F/A190S/V2 13A/S218V/A226R	193				
C22S/P38Q/K40M/F41R/L99N/ V138I/F173Y/D175W/A190S/V 213A/S218T/A226R	500				
C22S/P38Q/K40L/F41R/L99Y/ V138I/F173Y/D175N/A190S/V 213A/S218V/A226R	57				

Mutation String	fB Cleavage EC ₅₀ (nM)	fB Cleavage, 80% plasma EC ₅₀ (nM)	fB Cleavage , 64% plasma EC ₅₀ (nM)	fB Cleavage, 64% A ₂ M- depl plasma EC ₅₀ (nM)	t _{1/2} in 50% human plasma (min) (EGVD'A E)(SEQ ID NO: 13)
C22S/P38Q/K40M/F41R/L99S/ V138I/F173Y/D175N/A190S/V 213A/S218V/A226R	472				
C22S/P38Q/K40A/F41R/L99H/ V138I/D175N/A190S/V213A/S 218I/A226R	170				
C22S/P38Q/K40M/F41R/V138I/ F173S/D175Y/A190S/V213A/S 218V/A226R	287				
C22S/P38Q/K40M/F41R/V138I/ F173L/D175R/A190S/V213A/S 218T/A226R	51				
C22S/P38Q/K40L/F41R/V138I/ F173Y/D175R/A190S/V213A/S 218T/A226R	223				
C22S/P38Q/K40A/F41K/L99N/ V138I/D175H/A190S/V213A/S 218V/A226R	121				
C22S/P38Q/K40M/F41R/V138I/ F173Y/D175Y/A190S/V213A/S 218I/A226R	465				

Table 10B: MTSP-1-, uPA-, and Chymase-Based Engineered Proteases Cleavage Assays for EC₅₀ Factor B

<i>Scaffold</i>	<i>Mutation String</i>	hu Factor B cleavage (ELISA) EC ₅₀ (nM)		
		<i>n</i>	<i>Average</i>	<i>±S.D.</i>
uPA	V38D/A96E/D97G/C122S/Y172L/A98G/H99 M/T97a_L97bdel	1	no cleavage @ 1.2 uM	
uPA	V38D/C122S/T97a_L97bdel	1	no cleavage @ 3 uM	
uPA	V38D/A96G/D97E/A98G/H99V/C122S/T97a _L97bdel	1	no cleavage @ 0.95 uM	
uPA	C122S/V38D/A96N/D97A/A98G/H99V/T97a _L97bdel	1	no cleavage @ 1 uM	

<i>Scaffold</i>	<i>Mutation String</i>	hu Factor B cleavage (ELISA) EC ₅₀ (nM)		
		<i>n</i>	<i>Average</i>	<i>±S.D.</i>
uPA	V38D/C122S/D97L/A98G/H99V/T97a_L97b del	1	no cleavage @ 0.95 uM	
uPA	V38D/C122S/A96E/D97R/A98G/H99V/T97a _L97bdel	1	no cleavage @ 3 uM	
uPA	V38D/C122S/A96E/D97H/A98G/H99L/T97a _L97bdel	1	no cleavage @ 1.05 uM	
uPA	V38D/A96E/D97E/C122S/A98G/H99A/T97a _L97bdel	1	no cleavage @ 0.75 uM	
uPA	V38D/C122S/A96D/D97G/A98G/H99A/T97a _L97bdel	1	no cleavage @ 1.1 uM	
uPA	V38D/D97A/C122S/A98G/H99L/T97a_L97b del	2	6560.0	8145.9
uPA	V38D/C122S/A96D/D97W/A98N/H99L/T97a _L97bdel	1	3720.0	
uPA	V38D/C122S/A96D/D97T/A98G/H99M/T97a _L97bdel	1	7860.0	
uPA	V38D/A98G/H99A/C122S/T97a_L97bdel	1	no cleavage @ 0.8 uM	
uPA	V38D/A98G/H99A/C122S/L97bdel	1	no cleavage @ 1.6 uM	
uPA	V38D/A98G/H99L/C122S/T97a_L97bdel	1	no cleavage @ 0.55 uM	

[0346] Further, various chymase-based engineered proteases were tested to evaluate their baseline stability, by testing the effects of indirect and MTSP buffer (+/- EDTA) on the chymase-based engineered proteases. The peptide substrate EGVDAE-QF (SEQ ID NO: 52) was used for these experiments.

[0347] FIGS. 4A-4E depict graphs showing the stability of five different chymase proteases tested using a peptide substrate, in PBS, at 37°C. Table 11A summarizes the data shown in FIGS. 4A-4E, and lists the engineered proteases tested.

Table 11A: Activity of Chymase-based Engineered Proteases Tested with Peptide Substrate

Figure	Mutation of Engineered Protease	Fractional remaining activity						
		0 hr	2 hrs	4 hrs	6 hrs	24 hrs	72 hrs (Day 3)	168 hrs (Day 7)
FIG. 4A	C22S/K40L/F41K/L99N/F173Y/D175N/S218L/A226R	1.00	0.96	0.91	0.86	0.92	0.85	0.67
FIG. 4B	C22S/P38Q/V138I/A190S/V213A/A226R	1.00	0.96	0.91	0.86	0.92	0.85	0.67
FIG. 4C	C22S/P38Q/V138I/A190S/V213A/A226R	1.00	1.02	1.01	1.02	1.01	1.02	0.85
FIG. 4D	C22S/P38Q/K40L/F41R/V138I/F173Y/D175N/A190S/V213A/S218V/A226R	1.00	0.97	0.99	0.95	0.92	0.91	0.77
FIG. 4E	C22S/P38Q/K40M/F41R/V138I/F173Y/D175R/A190S/V213A/S218V/A226R	1.00	0.94	0.92	0.82	0.67	0.60	0.47

[0348] As shown, these proteases were more stable in the indirect buffer having a pH of 7.4. Wild type or naturally occurring chymase showed a loss of activity in all buffers, and EDTA had no apparent effect on stability.

[0349] Next, the second order rate constant (k^*) was measured for the inhibition of various non-naturally occurring chymase-based proteases by plasma, for the extrapolation of the half-life in 100% plasma. Briefly, a baseline chymase having the modifications C22S/A226R was used. The concentration of the baseline chymase and the tested chymase-based engineered proteases, listed in Table 11B below, were used at 50 nM, and another tested chymase C22S/L99R/F173Y/K192R/S218L/A226R was used at 25 nM. For the baseline chymase, a time course of protease activity in the presence of plasma was measured using 4.2 μ M CPQ2-ITLLSA-K(5FAM)-K-PEG8-K(biotin)-NH₂ (SEQ ID NO: 17) / 21 μ M Neutravidin at 37°C. A summary of the results is presented in Table 11B below.

Table 11B: Second Order Rate Constant Measurements and Calculated $t_{1/2}$

Mutation string	k^* ($\%^{-1}s^{-1}$)	calculated $t_{1/2}$ (s) (in 100% plasma)
C22S/A226R	7.32E-5	95
C22S/L99R/F173Y/K192R/S218L/A226R	6.24E-5	111
C22S/L99Y/F173Y/K192R/S218L/A226R	3.71E-5	187

Example 4: Factor B Add-Back Hemolysis Assay

[0350] Hemolysis assays were performed to evaluate the hemolytic activity of various lots of Factor B, a lot obtained from CompTech as compared to a lot expressed in-house. Briefly, Factor B depleted human serum was used, and plasma purified or recombinant human Factor B was used for the add-back. Rabbit red blood cells (RBCs) or chicken RBCs were washed in GVB/Mg/EGTA, and the depleted serum was spiked with Factor B. The RBC lysis was then monitored with CVF/FD/human Factor B convertase. These were analyzed to create standard curves from which the Factor B lots were compared. FIGS. 5-6 depict a bar graph of controls used for the hemolysis assay and the standard curves measured from the tested engineered proteases from the Factor B add-back hemolysis assay, respectively. Table 12 below presents a summary of the data from engineered proteases based on MTSP-1 and uPA. FIG. 6 also shows that the MTSP-1- based engineered protease F97E/K224N/F99L/D217I/C122S/C[17]S/C[19]S is able to inhibit hemolysis. This engineered protease was tested at two concentrations (samples 1 and 2). All references in this Example to engineered proteases are depicted in chymotrypsin numbering. The chymotrypsin numbering key for the modified protease domains of engineered proteases are found in Tables 2, 4, and 6 for uPA, MTSP-1, and chymase, respectively.

Table 12: Factor B Add-Back Hemolysis Data Summary

Scaffold	Mutation String	$[Protease]_{high}$ (nM)	Hu fB Add-Back EC_{50} (nM)
MTSP-1	C122S	4000	480.4
		4000	304.0
		4000	916.7

Scaffold	Mutation String	[Protease] ^{high} (nM)	Hu fB Add-Back EC ₅₀ (nM)
MTSP-1		4000 400	37.3 32.2
MTSP-1	I41G/F97D/F99L/C122S	4000 4000 4000	266.9 108.6 333.9
MTSP-1	A77aT/F94Y/C122S/D125G	4000 4000	65.9 125.3
MTSP-1	I41A/F97D/F99L/C122S	4000	282.9
MTSP-1	I41G/F97D/F99L/C122S/Y146D/C[17]S/C[19]S	4000 4000	282.9 394.7
MTSP-1	I41G/F97E/F99L/C122S/C[17]S/C[19]S	4000 4000 4000	362.5 357.5 292.5
MTSP-1	I41N/F97D/F99L/C122S/C[17]S/C[19]S	4000	222.8
MTSP-1	F97L/T98G/F99V/C122S/C[17]S/C[19]S/- null D96insD	4000 4000	4593.0 3493.0

Example 5: Peptide Cleavage Assay (k_{cat}/K_M)

[0351] Peptide cleavage assays were performed to evaluate k_{cat}/K_m of various engineered proteases of the disclosure based on MTSP-1, uPA, or chymase. All references in this Example to engineered proteases are depicted in chymotrypsin numbering. The chymotrypsin numbering key for the modified protease domains of engineered proteases are found in Tables 2, 4, and 6 for uPA, MTSP-1, and chymase, respectively. Cleavage of various substrates was evaluated to determine k_{cat}/K_m for protease activity. Briefly, k_{cat}/K_m values were determined from the slopes of the linear portion of a plot of V_o as a function of substrate concentration. Generally, the following conditions were used: a protease concentration of 50 nM, a substrate concentration of a maximum of 20 μ M, and 1.5-fold serial dilutions, and a temperature of 30°C. For the calculations, a semi-automated Michaelis Menten Kinetics protocol with quadruplicate measurements was used.

[0352] For chymase-based engineered proteases, the following quenched fluorescence (QF) peptide substrates were used: TQ2-KDVFYQMKK-Lys(5FAM)-NH₂ (SEQ ID NO: 29), and TQ2-KDVFYQMKK-Lys(5FAM) (SEQ ID NO: 30). For MTSP-1- and uPA-based engineered proteases, the following peptide substrates were used: 5FAM-EQQKRKIVL-K(QXL520)-NH₂ (SEQ ID NO: 31), CPQ2-PEQQKR-K(5FAM)-NH₂ (SEQ ID NO: 32), TQ2-GEQQKRKIVL-

Lys(5FAM)-NH₂ (SEQ ID NO: 33), Ac-QQKR-ACC (SEQ ID NO: 34). Table 13A below presents the various substrates used to test for protease activity at specific cleavage site sequences, and Table 13B below presents the EGVDAE QF (SEQ ID NO: 52) substrate k_{cat}/K_M (M⁻¹s⁻¹) based on various proteases that were tested.

Table 13A: Peptide Substrates for Various Cleavage Site Sequences

Cleavage site amino acid sequence	Peptide substrates used
EGVD (SEQ ID NO: 35)	Ac-EGVD-ACC (SEQ ID NO: 39)
	CPQ2-EGVDAE-K(5FAM)-K-NH ₂ (SEQ ID NO: 40)
	Biotin-GGGLSSLTETIEGVDAE-K(TAMARA)-NH ₂ (SEQ ID NO: 41)
	CPQ2-EGVDAE-K(5FAM)-K-PEG8-K(Biotin)-NH ₂ (SEQ ID NO: 42)
QQKR (SEQ ID NO: 36)	CPQ2-PEQQKR-K(5FAM)-NH ₂ (SEQ ID NO: 32)
	TQ2-GEQQKRKIVL-Lys(5FAM)-NH ₂ (SEQ ID NO: 33)
	Ac-QQKR-ACC (SEQ ID NO: 34)
	5FAM-EQQKRKIVL-K(QXL520)-NH ₂ (SEQ ID NO: 31)
DVFY (SEQ ID NO: 37)	TQ2-KDVFYQMKK-Lys(5FAM)-NH ₂ (SEQ ID NO: 29)
	TQ2-KDVFYQMKK-Lys(5FAM) (SEQ ID NO: 30)
	Ac-WEHR-ACC (SEQ ID NO: 43)
KNQKR (SEQ ID NO: 38)	Ac-KNQK-ACC-TFA Salt (SEQ ID NO: 44)
	TQ2-KNQKRQKQ-Lys(5FAM)-NH ₂ (SEQ ID NO: 45)
	Ac-KNQK-ACC (SEQ ID NO: 46)
	WSHPQFEKKNQKRQKQ-K(5FAM)-NH ₂ (SEQ ID NO: 47)

Table 13B: k_{cat}/K_M for Chymase-Based Engineered Proteases Measured with EGVDAE-QF (SEQ ID NO: 52)

Mutation	EGVDAE QF (SEQ ID NO: 52) k_{cat}/K_M (M ⁻¹ s ⁻¹)
C22S/F173Y/D175N/A226R	2665
C22S/K40L/F41R/L99N/F173Y/S218L/A226R	197
C22S/K40L/F41H/L99N/F173Y/S218L/A226R	190

Mutation	EGVDAE QF (SEQ ID NO: 52) k_{cat}/K_M ($M^{-1}s^{-1}$)
C22S/K40L/F41K/L99N/F173Y/S218L/A226R	194
C22S/K40L/F41R/L99N/F173Y/D175N/S218L/A226R	793
C22S/K40L/F41H/L99N/F173Y/D175N/S218L/A226R	638
C22S/K40L/F41K/L99N/F173Y/D175N/S218L/A226R	721

[0353] The results of the peptide cleavage assays are presented in Tables 14A-14B below.

Table 14A: Peptide Cleavage Assay Data for uPA-Based Scaffold

Scaffold	Mutation String (Chymo #'s)	Specific Activity (μM substrate/min/ μM enzyme)			k_{cat}/K_M ($M^{-1}s^{-1}$) (AGR-ACC)			k_{cat}/K_M ($M^{-1}s^{-1}$) (GLAR-ACC) (SEQ ID NO: 49)		
		QQKR-ACC (SEQ ID NO: 48)			<i>n</i>	Average	$\pm S.D.$	<i>n</i>	Average	$\pm S.D.$
uPA	C122S	0.0			4	9.68E+04	1.37E+03	8	6.86E+03	1.60E+03
uPA	H99L/C122S							4	3.15E+04	4.81E+03
uPA	H99P/C122S							2	2.19E+05	8.64E+03
uPA	G18E/R36S/V38D/C122S/V159A	0.0						2	1.75E+03	6.06E+01
uPA	H99D/C122S							2	1.31E+03	6.45E+01
uPA	H99N/C122S	0.2						1	5.38E+03	
uPA	H99C/C122S	0.5								
uPA	C122S/R217V	0.1						1	6.81E+03	
uPA	R36H/S37dP/V38D/C122S/A184T							2	5.49E+02	1.42E+01
uPA	V38D/C122S/T97a_L97bdel				1	6.95E+03		1	4.90E+04	
uPA	V38D/A96G/D97E/A98G/H99V/C122S/T97a_L97bdel				1	5.27E+03		1	3.05E+04	
uPA	C122S/V38D/A96N/D97A/A98G/H99V/T97a_L97bdel				1	9.16E+03		1	3.12E+04	
uPA	V38D/C122S/D97L/A98G/H99V/T97a_L97bdel				1	5.77E+03		1	2.54E+04	

Scaffold	Mutation String (Chymo #'s)	Specific Activity (μM substrate/min/ μM enzyme)			$k_{\text{cat}}/K_{\text{M}}$ ($\text{M}^{-1}\text{s}^{-1}$) (AGR-ACC)			$k_{\text{cat}}/K_{\text{M}}$ ($\text{M}^{-1}\text{s}^{-1}$) (GLAR-ACC) (SEQ ID NO: 49)		
		QQKR-ACC (SEQ ID NO: 48)			<i>n</i>	Average	$\pm\text{S.D.}$	<i>n</i>	Average	$\pm\text{S.D.}$
uPA	V38D/C122S/A96E/D97R/A98G/H99V/T97a_L97bdel				1	1.32E+04		1	1.79E+04	
uPA	V38D/C122S/A96E/D97H/A98G/H99L/T97a_L97bdel				1	5.05E+04		1	4.21E+05	
uPA	V38D/A96E/D97E/C122S/A98G/H99A/T97a_L97bdel				1	1.04E+04		1	8.53E+03	
uPA	V38D/C122S/A96D/D97G/A98G/H99A/T97a_L97bdel				1	1.53E+04		1	8.49E+03	
uPA	V38D/D97A/C122S/A98G/H99L/T97a_L97bdel				1	7.60E+04		1	5.31E+05	
uPA	V38D/C122S/A96D/D97W/A98N/H99L/T97a_L97bdel				1	3.86E+04		1	2.52E+05	
uPA	V38D/C122S/A96D/D97T/A98G/H99M/T97a_L97bdel				1	4.79E+04		1	2.08E+05	

Table 14A: Continued: Peptide Cleavage Assay Data for uPA-Based Scaffold

Scaffold	Mutation String (Chymo #'s)	$k_{\text{cat}}/K_{\text{M}}$ ($\text{M}^{-1}\text{s}^{-1}$) (QQKR-ACC) (SEQ ID NO: 48)		
		<i>n</i>	Average	$\pm\text{S.D.}$
uPA	C122S	5	1.67E+02	3.05E+01
uPA	H99L/C122S	2	3.24E+03	7.27E+01
uPA	H99P/C122S	2	6.18E+03	3.05E+02
uPA	G18E/R36S/V38D/C122S/V159A	0		
uPA	H99D/C122S	2	6.00E+02	2.83E+01
uPA	H99N/C122S	1	3.68E+02	

Scaffold	Mutation String (Chymo #'s)	k_{cat}/K_m ($M^{-1}s^{-1}$) (QQKR-ACC) (SEQ ID NO: 48)		
		<i>n</i>	Average	$\pm S.D.$
uPA	H99C/C122S	2	2.30E+03	4.47E+02
uPA	C122S/R217V	2	8.42E+02	2.10E+02
uPA	R36H/S37dP/V38D/C122S/A184T	0		
uPA	V38D/A96E/D97G/C122S/Y172L/A98G/H99M/T97a_L97bdel	1	7.50E+03	
uPA	V38D/C122S/T97a_L97bdel	1	9.14E+03	
uPA	V38D/A96G/D97E/A98G/H99V/C122S/T97a_L97bdel	2	6.86E+04	8.34E+03
uPA	C122S/V38D/A96N/D97A/A98G/H99V/T97a_L97bdel	2	7.61E+04	3.87E+03
uPA	V38D/C122S/D97L/A98G/H99V/T97a_L97bdel	2	3.64E+04	5.64E+03
uPA	V38D/C122S/A96E/D97R/A98G/H99V/T97a_L97bdel	2	5.51E+04	5.48E+03
uPA	V38D/C122S/A96E/D97H/A98G/H99L/T97a_L97bdel	2	1.42E+05	3.50E+04
uPA	V38D/A96E/D97E/C122S/A98G/H99A/T97a_L97bdel	2	4.43E+04	2.65E+03
uPA	V38D/C122S/A96D/D97G/A98G/H99A/T97a_L97bdel	2	9.24E+04	2.03E+03
uPA	V38D/D97A/C122S/A98G/H99L/T97a_L97bdel	2	7.19E+04	2.26E+04
uPA	V38D/C122S/A96D/D97W/A98N/H99L/T97a_L97bdel	2	2.05E+05	5.39E+03
uPA	V38D/C122S/A96D/D97T/A98G/H99M/T97a_L97bdel	2	7.28E+04	1.47E+04
uPA	V38D/D97E/L97bV/A98G/H99V/C122S/A96_H99del	1	1.39E+04	
uPA	V38D/A96G/D97A/H99E/C122S/L97b_H99del	1	6.05E+02	
uPA	V38D/C122S/A96_H99delinsPGVE	1	6.74E+02	
uPA	V38D/C122S/L97b_H99del/D97delinsEG	1	1.39E+04	
uPA	V38D/T97aS/L97bV/A98T/H99E/C122S/A96_H99del	1	1.11E+03	
uPA	V38D/C122S/A96del/A98_H99del/-nulldelinsLK	1	2.77E+04	
uPA	V38D/A96G/D97E/A98G/H99E/C122S/T97a_H99del	1	5.74E+02	
uPA	V38D/C122S/A96del/A98_H99del/-nulldelinsRS	1	2.71E+04	
uPA	V38D/D97E/C122S/-null_H99delinsSPG	1	4.31E+03	
uPA	Y40H/C122S	0		
uPA	V38D/A98G/H99A/C122S/T97a_L97bdel	1	5.99E+04	
uPA	V38D/A98G/H99A/C122S/L97bdel	1	3.44E+03	
uPA	V38D/A98G/H99L/C122S/T97a_L97bdel	1	8.81E+04	

Table 14A: Continued: Peptide Cleavage Assay Data for uPA-Based Scaffold

Scaffold	Mutation String (Chymo #'s)	k_{cat}/K_m ($M^{-1}s^{-1}$) (QQKR/KI-QF) (SEQ ID NO: 50)		
		<i>n</i>	Average	$\pm S.D.$
uPA	C122S	0		
uPA	H99L/C122S	0		
uPA	H99P/C122S	0		
uPA	H99D/C122S	0		
uPA	V38D/A96E/D97G/C122S/Y172L/A98G/H99M/T97a_L97bdel	0		
uPA	V38D/C122S/T97a_L97bdel	0		
uPA	C122S/V38D/A96N/D97A/A98G/H99V/T97a_L97bdel	1	5.16E+02	
uPA	V38D/C122S/D97L/A98G/H99V/T97a_L97bdel	0		
uPA	V38D/C122S/A96E/D97R/A98G/H99V/T97a_L97bdel	1	4.73E+02	
uPA	V38D/C122S/A96E/D97H/A98G/H99L/T97a_L97bdel	1	1.45E+03	
uPA	V38D/A96E/D97E/C122S/A98G/H99A/T97a_L97bdel	0		
uPA	V38D/C122S/A96D/D97G/A98G/H99A/T97a_L97bdel	1	2.77E+02	
uPA	V38D/D97A/C122S/A98G/H99L/T97a_L97bdel	1	6.57E+02	
uPA	V38D/C122S/A96D/D97W/A98N/H99L/T97a_L97bdel	1	3.09E+03	
uPA	V38D/C122S/A96D/D97T/A98G/H99M/T97a_L97bdel	1	9.75E+02	
uPA	V38D/C122S/A96del/A98_H99del/-nulldelinsLK			

Table 14B: Peptide Cleavage Assay Data for MTSP-1-Based and Chymase-Based Scaffold

Scaffold	Mutation String (Chymo #'s)	Specific Activity (uMsubstrate/ min/uMenzyme)		k_{cat}/K_m ($M^{-1}s^{-1}$) (QHRR-ACC) (SEQ ID NO: 51)		
		QQKR-ACC (SEQ ID NO: 48)		<i>n</i>	Average	$\pm S.D.$
MTSP	C122S	2.6		1	3.39E+03	
MTSP	I41G/F97D/F99L/C122S			1	1.07E+06	
MTSP	A77aT/F94Y/C122S/D125G			1	4.08E+04	
MTSP	I41A/F97D/F99L/C122S			1	5.35E+05	
MTSP	I41S/F97D/F99V/C122S/Y146D/C[17]S/C[19]S			1	1.06E+05	
MTSP	I41G/F97D/F99L/C122S/Y146D/C[17]S/C[19]S			1	6.87E+05	
MTSP	I41G/F97E/F99L/C122S/C[17]S/C[19]S			1	7.72E+05	
MTSP	I41N/F97D/F99L/C122S/C[17]S/C[19]S			1	8.05E+05	
MTSP	I41A/F97D/F99V/C122S/Y146D/Q175T/C[17]S/C[19]S			1	5.03E+04	
MTSP	I41G/F97D/F99M/C122S/C[17]S/C[19]S			1	2.41E+05	
MTSP	F97L/T98G/F99V/C122S/C[17]S/C[19]S/- null_D96insD			1	2.62E+04	

Table 14B: Continued: Peptide Cleavage Assay Data for MTSP-1-Based and Chymase-Based Scaffold

Scaffold	Mutation String (Chymo #s)	k_{cat}/K_m ($M^{-1}s^{-1}$) (QOKR-ACC) (SEQ ID NO: 48)			k_{cat}/K_m ($M^{-1}s^{-1}$) (RQAR-ACC) (SEQ ID NO: 54)			k_{cat}/K_m ($M^{-1}s^{-1}$) (angiotensinogen-QF)		
		<i>n</i>	Average	$\pm S.D.$	<i>n</i>	Average	$\pm S.D.$	<i>n</i>	Average	$\pm S.D.$
MTSP	C122S	9	2.28E+04	1.28E+04	7	3.89E+06	8.69E+05			
MTSP	F97E/K224N/F99L/D217I/C122S/C[17]S/C[19]S	2	1.49E+06	4.24E+05	1	2.31E+07				
MTSP	C[17]S/C[19]S/F97E/F99L/C122S/Q175L/Q192M/D217I/K224R	2	1.15E+06	2.05E+05	1	2.36E+07				
MTSP	C[17]S/C[19]S/F97M/F99L/C122S/Q175W/D217I/K224A	1	2.90E+06		1	3.72E+07				
MTSP	I41G/F97D/F99L/C122S	1	1.23E+05		1	4.91E+06				
MTSP	A77aT/F94Y/C122S/D125G	1	1.14E+05		1	4.27E+06				
MTSP	I41A/F97D/F99L/C122S	1	9.48E+04		1	3.92E+06				
MTSP	I41S/F97D/F99V/C122S/Y146D/C[17]S/C[19]S	1	5.13E+04		1	3.85E+05				
MTSP	I41G/F97D/F99L/C122S/Y146D/C[17]S/C[19]S	1	5.05E+04		1	2.66E+06				
MTSP	I41G/F97E/F99L/C122S/C[17]S/C[19]S	1	1.08E+05		1	3.50E+06				
MTSP	I41N/F97D/F99L/C122S/C[17]S/C[19]S	1	1.24E+05		1	2.39E+06				
MTSP	I41A/F97D/F99V/C122S/Y146D/Q175T/C[17]S/C[19]S	1	3.79E+04		1	1.97E+05				
MTSP	I41G/F97D/F99M/C122S/C[17]S/C[19]S	1	3.45E+04		1	1.28E+06				
MTSP	F97L/T98G/F99V/C122S/C[17]S/C[19]S/-null_D96insD	1	1.08E+04		1	2.17E+04				
MTSP	C122S/C[17]S/C[19]S/-null_T98insA	1	1.17E+04		3	3.38E+05	1.47E+04			

Scaffold	Mutation String (Chymo #s)	k_{cat}/K_m ($M^{-1}s^{-1}$) (QQR-ACC) (SEQ ID NO: 48)			k_{cat}/K_m ($M^{-1}s^{-1}$) (RQAR-ACC) (SEQ ID NO: 54)			k_{cat}/K_m ($M^{-1}s^{-1}$) (angiotensinogen-QF)		
		<i>n</i>	Average	$\pm S.D.$	<i>n</i>	Average	$\pm S.D.$	<i>n</i>	Average	$\pm S.D.$
MTSP	C122S/C[17]S/C[19]S/-null_T98insAA	1	1.92E+03		3	5.27E+05	3.26E+05			
MTSP	C[17]S/C[19]S/I41Q/F97T/F99L/C122S/Y146D/Q175W	2	4.96E+05	3.20E+04	1	1.85E+07				
MTSP	C[17]S/C[19]S/I41F/F97D/F99I/C122S/Y146D/Q175S/D217T	2	1.19E+05	1.19E+05	1	8.78E+06				
MTSP	C[17]S/C[19]S/I41R/F97D/F99N/C122S/Y146D/D217V/K224Q	2	1.64E+04	8.81E+03	1	1.11E+05				
MTSP	C[17]S/C[19]S/I41R/F97E/F99N/C122S/Y146D/D217Y/K224M	2	1.70E+04	8.13E+03	1	1.76E+05				
MTSP	C[17]S/C[19]S/I41G/F97E/F99L/C122S/R161G/D217Y/K224Q	2	3.61E+04	2.62E+04	1	1.05E+07				
MTSP	C[17]S/C[19]S/I41R/F97D/F99H/C122S/Y146D/Q175P/Q192M/D217F/K224L	1	2.09E+03		1	6.67E+03				
MTSP	C[17]S/C[19]S/I41R/F97E/F99S/C122S/Y146D/Q175R/D217I/K224M	2	1.33E+04	1.11E+04	1	4.45E+04				
MTSP	C[17]S/C[19]S/I41K/F97D/F99Y/C122S	2	5.62E+02	1.98E+01	1	7.04E+04				
MTSP	I41K/F97D/F99H/C122S/C[17]S/C[19]S	2	5.49E+02	1.27E+02	1	7.71E+03				
MTSP	F94Y/F99L/C122S/G197A/C[17]S/C[19]S	1	6.73E+05		1	4.58E+06				
MTSP	F99L/C122S/L172M/G197A/C[17]S/C[19]S	1	1.72E+05		1	9.70E+06				
MTSP	D23Y/L70Q/F99L/K110I/C122S/V183A/A204V/C[17]S/C[19]S	1	4.91E+05		1	1.12E+07				
MTSP	F94Y/C122S/C[17]S/C[19]S	2	2.23E+05	4.54E+03	1	7.10E+06				

Scaffold	Mutation String (Chymo #s)	k_{cat}/K_m ($M^{-1}s^{-1}$) (QQR-ACC) (SEQ ID NO: 48)			k_{cat}/K_m ($M^{-1}s^{-1}$) (RQAR-ACC) (SEQ ID NO: 54)			k_{cat}/K_m ($M^{-1}s^{-1}$) (angiotensinogen-QF)		
		<i>n</i>	Average	$\pm S.D.$	<i>n</i>	Average	$\pm S.D.$	<i>n</i>	Average	$\pm S.D.$
chymase	C22S							2	1.69E+05	1.14E+03
chymase	C22S/L99G							1	1.73E+04	
chymase	C22S/F173L							1	1.29E+05	
chymase	C22S/L99G/F173L							1	1.17E+04	

Table 14B: Continued: Peptide Cleavage Assay Data for MTSP-1-Based and Chymase-Based Scaffold

Scaffold	Mutation String (Chymo #s)	k_{cat}/K_m ($M^{-1}s^{-1}$) (DVFY/QM-QF) (SEQ ID NO: 55)			k_{cat}/K_m ($M^{-1}s^{-1}$) (NVFY/QM-QF) (SEQ ID NO: 56)		
		<i>n</i>	Average	$\pm S.D.$	<i>n</i>	Average	$\pm S.D.$
chymase	C22S	2	1.79E+05	4.99E+03	3	1.22E+05	5.17E+04
chymase	C22S/L99G	1	4.93E+05		1	7.20E+05	
chymase	C22S/F173L	1	1.34E+05		1	1.32E+05	
chymase	C22S/L99G/F173L	1	3.59E+05		1	7.78E+05	

Example 6: Inhibition Tests for Engineered proteases

[0354] Inhibition tests were carried out using chymase-based engineered proteases. Various serpins, which are capable of inhibiting protease activity, were used to test whether these could be used for adequate inhibition of the tested chymase-based engineered proteases, to enable selection of an engineered protease resistant to inhibition in plasma. A summary of the serpin inhibition tests are provided in Table 15 below. All references in this Example to engineered proteases are depicted in chymotrypsin numbering. The chymotrypsin numbering key for the modified protease domains of engineered proteases are found in Tables 2, 4, and 6 for uPA, MTSP-1, and chymase, respectively.

Table 15: Serpin Inhibition Summary

Mutation String	Plasma Inhibitor	$k_{app}(M^{-1}s^{-1})$
C22S	Plasma antichymotrypsin (ACT)	31166
C22S/A226R	alpha-2-macroglobin (A ₂ M)	812
	ACT	1045
C22S/F41I/L99W/G151L/F173K/R217S/A226R	A ₂ M - YESD/VM	530 - 37
C22S/F41V/L99W/G151L/F173K/R217S/A226R	A ₂ M	329
C22S/L99H/F173Y/K192R/S218L/A226R	A ₂ M	356
C22S/L99Y/F173Y/K192R/S218L/A226R	A ₂ M	3127
C22S/F173Y/D175N/A226R	A ₂ M	645
	ACT	428
C22S/K40L/F41R/L99N/F173Y/S218L/A226R	plasma ACT	190
C22S/K40M/F41R/L99N/G151S/F173Y/S218L/A226R	plasma ACT	317
C22S/K40L/F41H/L99N/F173Y/S218L/A226R	plasma ACT	193
C22S/K40L/F41L/L99N/G151H/F173Y/S218V/A226R	plasma ACT	252
C22S/K40L/F41K/L99H/G151S/F173Y/S218L/A226R	plasma ACT	139
C22S/K40L/F41K/L99H/F173Y/S218L/A226R	plasma ACT	147
C22S/K40L/F41K/L99N/F173Y/S218L/A226R	plasma ACT	197
C22S/A190S/A226R	plasma ACT	1599
C22S/S189T/A190S/A226R	plasma ACT	935
C22S/K40L/F41R/L99N/F173Y/D175N/S218L/A226R	plasma ACT	37
C22S/K40L/F41H/L99N/F173Y/D175N/S218L/A226R	plasma ACT	40
C22S/K40L/F41K/L99N/F173Y/D175N/S218L/A226R	plasma ACT	28
C22S/P38Q/V138I/V183I/L199V/A226R/F228V	plasma ACT	269
C22S/K40M/F41R/L99Q/F173Y/D175R/S218I/A226R	46 min (disc)	

Example 6: Factor B Cleavage and Activity of KLK5 Protease

[0355] FIG. 7A depicts a graph showing hemolysis inhibition by KLK5 and compared with a chymase-based engineered protease C22S/F173Y/D175N/A226R. All references in this Example to engineered proteases are depicted in chymotrypsin numbering. The chymotrypsin numbering key for the modified protease domains of engineered proteases are found in Tables 2, 4, and 6 for uPA, MTSP-1, and chymase, respectively. Briefly, a digestion reaction was prepared with 4.0 μ M of Human Factor B (Complement Technologies) in 20 μ L buffer (50 mM Tris pH 7.4/50 mM NaCl/0.01% Tween 20). Different concentration of the KLK5 protease or the

chymase-based engineered protease C22S/F173Y/D175N/A226R (400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, and 0.39 with a control at 0.0 nM) were added to 4 μ M of Factor B and incubated for 1 hour at 37°C. After digestion, 6 μ L of the reaction was evaluated in a hemolysis assay.

[0356] The Factor B digestion reactions were diluted in 20 μ L of GVB (Complement Technologies), 10 mM MgCl_2 and 8 mM EGTA (GVB/Mg/EGTA). 45 μ L of human Factor B Depleted Serum (Complement Technologies) was then added to 5 μ L of human Factor B digests to obtain a final volume of 90% serum. In parallel, 50 μ L of rabbit red blood cells (Colorado Serum Co.) were diluted into 950 μ L of GVB/Mg/EGTA and mixed gently. After spinning at 2000 RPM for 5 minutes at 4°C the rabbit cells were resuspended in 1 ml of GVB/Mg/EGTA. The washed rabbit cells were then incubated with the mixture of serum + Factor B digests in GVB/Mg/EGTA buffer to obtain a 15% serum final concentration and incubated for 1 hour at 37°C under agitation. The reaction was then centrifuged at 2000 RPM for 5 minutes and 100 μ L of the supernatant was transferred to a clear, flat-bottomed 96 well plate. The plate absorbance was read at 415 nm with a spectrophotometer and the EC_{50} was calculated. The results of the hemolysis assay are depicted in FIG. 7A, showing that the KLK5 protease was effective in inhibition of hemolysis.

[0357] FIG. 7B depicts a graph showing Factor B cleavage with KLK5 and a chymase-based engineered protease C22S/F173Y/D175N/A226R. Briefly, A digestion reaction is prepared with 4.0 μ M of Human Factor B (Complement Technologies) in 20 μ L buffer (50 mM Tris pH 7.4/50 mM NaCl/0.01% Tween 20). Different concentrations of KLK5 (400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, and 0.39 with a control at 0.0 nM) are added to 4 μ M of factor B and incubated for 1 hour at 37°C. After digestion, 15 μ L of the reaction is transferred to a 96 well plate with 1.5 μ L of 0.2N HCL. After quenching, the reactions are prepared factor B ELISA.

[0358] A Factor B standard curve is made with 800, 533.3, 355.5, 237.0, 158.0, 105.3, 70.2, 46.8, 31.2, 20.8, 0.0 pM in 1% BSA-PBST. A 384 well plate was coated with the monoclonal antibody against human Factor Ba (#A225, Quidel) at 2 μ g/mL in carbonate buffer (25 μ L/well). After blocking for 1 hour at room temperature with 1% BSA-PBST (100 μ L/well), the digests (chymase + Factor B) were diluted to 800 pM and standards were diluted 1:1.5 from 800 pM into the blocking buffer (25 μ L/well). The plate was agitated for 30 minutes at room temperature.

[0359] The biotinylated monoclonal antibody against human Factor Bb (Quidel) was then added at 0.125 µg/ml to detect the bound Factor B. Streptavidin-HRP was then diluted at 1:200 in blocking buffer (25 µL/well) and the plate was agitated for 30 minutes at room temperature. The plate was developed with ELISABright, (50 µL/well) for 1 minute at room temperature and read in the EnVision plate reader. FIG. 7B depicts two independent experiments with different stocks of KLK5. These results show that the KLK5 protease was effective in cleavage Factor B, with comparable activity at the various concentrations used.

[0360] Factor B cleavage by KLK5 was also evaluated by Coomassie gel. FIGS. 8A-8B depict Coomassie gels showing examples of Factor B cleavage with KLK5 and a chymase-based engineered protease C22S/F173Y/D175N/A226R. Briefly, a digestion reaction was prepared with 4.0 µM of Human Factor B (Complement Technologies) in 20 µL buffer (50 mM Tris pH 7.4/50 mM NaCl/0.01% Tween 20). Different concentrations of KLK5 or the chymase-based engineered protease C22S/F173Y/D175N/A226R (400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, and 0.39 with a control at 0.0 nM) were added to 4 µM of Factor B and incubated for 1 hour at 37°C. After digestion, 15 µL of the reaction was transferred to a 96 well plate with 1.5 µL of 0.2N HCL. After quenching, the reactions were prepared for SDS-PAGE gel. 20 µL of the reaction mixtures were loaded per well of a 4-12% Bis-Tris Criterion gel. Densitometry analysis of the Factor B cleavage was performed, and EC₅₀ was calculated.

[0361] FIG. 9 depicts mass spectrometry (MS) data identifying the cleavage site at ²³⁴Arg within the QQKR/KIV (SEQ ID NO: 9) cleavage site of Factor B by KLK5, and identifying the cleavage site at ²²¹Asp within the EGVDAE (SEQ ID NO: 13) cleavage site of Factor B by the chymase-based engineered protease C22S/A226R. Briefly, human Factor B (Comptech) was incubated at 2 µM with different concentration of Kallikrein 5 (KLK5, R&D Systems) or the chymase-based engineered protease C22S/A226R at 10 nM or 100 pM for 10 minutes to 1 hour at 37°C in 20 mM Tris pH 8 buffer with 16-O or 18-O water. The reaction was quenched with 20 µM of inhibitor FFR-CMK for 30 minutes at room temperature. Half of the sample was further treated with Rapigest/chymotrypsin and adjusted to pH 3 by the addition of 1 mL 1% TFA. TCEP (100 mM final concentration) was then added to reduce disulfides for 30 minutes at 37°C. 12 mL of each sample was bound to a Ziptip (Millipore) and eluted with 15 mL 80% ACN-0.1% TFA. After drying in a Speedvac, the samples were re-dissolved in 4 mL of 30% ACN-0.05% TFA. 0.35 mL of the sample was loaded with 0.45 mL of CHCA matrix (10 mg/mL) onto an

OptiPlate, and analyzed by MALDI-MS (ABI 4700) in both linear (m/z 2-22k) and reflector (m/z 1500-5400) mode. The tandem MS was carried out on peptides of interest when possible. Two large peptide fragments were detected at ~33 and 59 kDa. In summary, 100 pM KLK5 cleaves at 1 of 38 Arg in a 10 minute reaction and in 4 out of 38 Arg in a 60 minute reaction (Arg175, Arg193, Arg730 and Arg739). At 10 nM, cleavage was detected in 18 of 38 Arg in 10 and 60 minute reactions (Arg50, Arg74, Arg94, Arg175, Arg182, Arg193, Arg202, Arg203, Arg259, Arg381, Arg415, Arg658, Arg679, Arg708, Arg710, Arg730, Arg739). The earliest cleavage event was at Arg 730.

[0362] The reconstructed ion chromatograms shown in FIG. 9 are of reaction products from Kallikrein-5 (“KLK”), the chymase-based engineered protease C22S/A226R (“chymase-based”), and plasma derived-Factor B (“Fb control”). These chromatograms show that the chymase-based engineered protease C22S/A226R cleaves Factor B at ²²¹Asp, wherein the peak at 3.8 minutes corresponding to intact chymase, the peak at 4.73 minutes corresponds to intact Factor B (739 residues with four A2 glycans, MW 91802), and the peak at 4.63 minutes corresponds to cleavage at ²²¹Asp as residues 222-739 containing two A2 glycans (MW 62827). In contrast, KLK5 cleaves Factor B at ²³⁴Arg (peak at 4.69 minutes, MW 61437). No remaining intact Factor B was present in this sample (top trace).

Example 7: Complement Activation and Cytokine Release Measured in Mouse Model of Acute Respiratory Distress Syndrome

[0363] FIG. 10 is a schematic depicting the general method for measuring complement activation and cytokine release from tissues of a mouse model of acute respiratory distress syndrome (ARDS) treated with a chymase-based engineered protease. All references in this Example to engineered proteases are depicted in chymotrypsin numbering. The chymotrypsin numbering key for the modified protease domains of engineered proteases are found in Tables 2, 4, and 6 for uPA, MTSP-1, and chymase, respectively. Briefly, mice received injections of a chymase-based engineered protease C22S/P38Q/K40L/F41R/V138I/F173Y/D175N/A190S/V213A/S218V/A226R, and lung function were measured, and post-sacrifice, bronchoalveolar lavage fluid was measured. In FIG. 10, the engineered protease is designated as “protease.”

[0364] After an adaptation period, each animal was weighed and randomly assigned to a treatment group based on body weight. On day 0 (0 hours), mice were anaesthetized and received a single intratracheal instillation (IT) of Lipopolysaccharide (LPS, Sigma) at a dosage of 50 µg per mouse. Control mice received an instillation of sterile 0.9% saline (50 µl). All animals were monitored for general health status and body weight over the disease course. Respiratory functions were measured by whole-body plethysmography (WBP) on conscious mice at 0, 6, 24, and 48 hours post-LPS IT. Three (3) hours post-LPS IT, mice received an intravenous (IV) injection of the chymase-based engineered protease C22S/P38Q/K40L/F41R/V138I/F173Y/D175N/A190S/V213A/S218V/A226R at 5 mg/kg or 6.5 mg/kg and control animals received an IV injection of vehicle (PBS). A subset of mice received a second IV injection of the chymase-based engineered protease C22S/P38Q/K40L/F41R/V138I/F173Y/D175N/A190S/V213A/S218V/A226R at 5 mg/kg or 6.5 mg/kg, 27 hours post-LPS IT (i.e. 24 hours post-first IV injection). Mice were sacrificed at 24 and 48 hours post-LPS IT. To obtain plasma, blood was drawn by facial puncture under anesthesia and collected in K₂EDTA microtainer tubes. After centrifuging at 2,000 x g for 10 minutes at 4°C and the plasma was aliquoted (60 µl) and stored at -80°C for future cytokine and complement analysis.

[0365] Next, a tracheotomy was performed to expose the lungs. The trachea was connected to a cannula and the left lung was clamped while 0.9 ml of cold PBS 1X, Protease Inhibitor 1X (SigmaFAST®) solution (3 x 300 µL) was injected to perform a bronchoalveolar lavage fluid (BALF) on the right lobe of the lungs. A first aliquot (300 µL) was kept for BALF total cells count with cells differential count. Two other aliquots of 60 µL each were stored at -80° for future complement and cytokine analysis. The right lung was immediately snap frozen and stored at -80°C for complement and cytokine analysis in the lung homogenate. The lung was homogenized in 1X PBS + 0.1% Triton X-100 with protease cocktail inhibitors to obtain a homogenate of 20 mg/100 µL and centrifuged at 2,520 x g for 15 minutes at 4°C. The supernatant was then processed for cytokine analysis.

[0366] Three hours post injection with the chymase-based engineered protease C22S/P38Q/K40L/F41R/V138I/F173Y/D175N/A190S/V213A/S218V/A226R, plethysmography measurements showed a significant reduction in the PenH values suggesting an improvement in lung function. However, this effect is not sustained over time (24 hour and

48 hour measurements). Later, 48 hours post-LPS, animals receiving 2 doses of the chymase-based engineered protease

C22S/P38Q/K40L/F41R/V138I/F173Y/D175N/A190S/V213A/S218V/A226R at 5-6.5 mg/kg showed a significant improvement of body weight suggesting this engineered protease reduced the severity of ARDS symptoms. At sacrifice 48 hours after LPS, animals receiving 2 doses of the chymase-based engineered protease at 5-6.5 mg/kg showed a trend for a lower neutrophil to lymphocyte ratio in BALF indicating that inflammatory infiltrates are reduced with the chymase-based engineered protease

C22S/P38Q/K40L/F41R/V138I/F173Y/D175N/A190S/V213A/S218V/A226R. These results are depicted in FIGS. 11A-11C. FIG. 11A depicts the pulmonary congestion index shown by the PenH value. FIG. 11B depicts body weight loss measured in the tested animals. FIG. 11C depicts the BALF Neutrophil-to-Lymphocyte Ratio (NLR) measured in the tested animals. The data shown are mean $\pm$ SEM, and *p values are < 0.05 using the Student's T-test. These results show the chymase-based engineered protease

C22S/P38Q/K40L/F41R/V138I/F173Y/D175N/A190S/V213A/S218V/A226R is efficacious in improving respiratory function in a mouse model of ARDS. In FIGS. 11A-11C, this engineered protease is designated as "protease."

[0367] FIGS. 12A-12D depict the results of BALF and lung cytokine measurement from mouse tissue after treatment with a chymase-based engineered protease

C22S/P38Q/K40L/F41R/V138I/F173Y/D175N/A190S/V213A/S218V/A226R. In FIGS. 12A-12D, this engineered protease is designated as "protease." Briefly, BALF and lung tissues were collected similarly as described above, 24 hours after a single dose of the chymase-based engineered protease

C22S/P38Q/K40L/F41R/V138I/F173Y/D175N/A190S/V213A/S218V/A226R was administered intravenously at 5 mg/kg 3 hours post-LPS. Tissues were processed for quantification of cytokines/chemokines using a Mouse Cytokine Array / Chemokine Array 31-Plex (MD31) measuring Eotaxin, G-CSF, GM-CSF, IFN γ , IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1 α , MIP-1 β , MIP-2, RANTES, TNF α , and VEGF (Eve Technologies). Samples were centrifuged prior to aliquoting and diluted 2-fold prior to running the assay according to Eve Technology protocol. IL-2, IL-6 and CXCL9 are significantly reduced in mouse bronchoalveolar fluids (BALF) and IL-6 is significantly reduced in lung

tissues indicating a lower pulmonary inflammation which may lead to a subsequent reduction in chemoattraction of inflammatory cells into lung tissues. Values are mean $\pm$ SEM and * $p < 0.05$, ** $p < 0.01$ using Student's *t* test. These results show that this engineered protease is efficacious in reducing inflammatory cytokines IL-2 and IL-6, and chemokine CXCL9 in a mouse model of ARDS.

Example 8: Activity Assessment and Characterization of a Mammalian Expressed Chymase-Based Engineered Protease

[0368] The ability for a chymase-based engineered protease zymogen (C22S/P38Q/K40A/F41R/L99H/V138I/F173Y/D175N/A190S/V213A/S218I/A226R) (Table 7B) (produced in a mammalian culture system, and purified and activated) to cleave CFB was assessed. All references in this Example to engineered proteases are depicted in chymotrypsin numbering. The chymotrypsin numbering key for the modified protease domains of engineered proteases are found in Tables 2, 4, and 6 for uPA, MTSP-1, and chymase, respectively. Enzymatic cleavage of Complement Factor B (CFB, Complement Technologies Cat# A135) was assessed via an AlphaLISA assay. Following *in vitro* cleavage at 37°C for 1 hour, reactions were diluted and transferred to a multi-well plate for bead based detection of CFB. For detection of CFB, an anti-Factor Ba antibody (Quidel Cat# A225) labeled with DIG (Biotium Mix N' Stain Kit, Cat# 92450) was paired with anti-DIG acceptor beads (Perkin Elmer Cat# AL113C) and a biotinylated anti Factor-Bb antibody (Quidel Cat# A712) was paired with streptavidin donor beads (Perkin Elmer, Cat# 6760002S). If non cleaved CFB remains, the acceptor and donor beads are brought together and upon laser excitation, a singlet oxygen from the donor bead drives a chemiluminescent signal from the acceptor bead that activates fluorophores contained in the same acceptor bead. Upon CFB cleavage by the engineered protease tested in this example, the resulting Ba and Bb cleavage products are no longer associated and a loss or reduction in signal results. Cleavage of full length CFB was quantified by linear regression against a CFB standard curve in the absence of chymase. Table 16 shows the results of the cleavage of CFB using the listed engineered protease.

Table 16: Cleavage of Complement Factor B (CFB)

Engineered Chymase-Based Protease	Assay	Metric	Measurement
C22S/P38Q/K40A/F41R/L99H/V138I/F173Y/D175N/A190S/V213A/S218I/A226R	AlphaLI SA	EC ₅₀	190-212 nM

[0369] The engineered protease was also assessed for inhibition of hemolysis both in a standard Alternative Pathway (AP) hemolysis assay as well as in an enhanced version of the assay, similar to that described in Example 4. Hemolysis inhibition by the small molecule Factor B inhibitor LNP023 (Iptacopan, MedChemExpress Cat# HY-127105) was assessed at in the same experiments as a comparison. For the standard AP assay, varying concentrations of the C22S/P38Q/K40A/F41R/L99H/V138I/F173Y/D175N/A190S/V213A/S218I/A226R engineered protease or LNP023 or relevant vehicle controls were premixed with 20% Normal Human Serum (NHS) for 10 minutes at 37°C. Rabbit red blood cells (RBCs) were then added along with Alternative Pathway buffer (gelatin veronal buffer, GVB +Mg +EGTA, 0.1% gelatin, 5mM Veronal, 145 mM NaCl, 0.025% NaN₃, pH 7.3, 10 mM MgCl₂ and 8 mM EGTA) and incubated for 30 minutes at 37°C. Cells were then pelleted and supernatant OD at 415 nm measured to assess lysis. For the enhanced version of the assay the following adjustments were made: NHS was replaced with human Factor B depleted sera (Complement Technologies Cat#A335), Factor B purified from human serum was spiked back into the serum for a final concentration in the hemolysis assay of 1.6 mM, and preincubation of the serum with drug was carried out for 180 min. at 37°C. Percent lysis was calculated using the following formula: $[(OD_{415} \text{ of sample} - OD_{415} \text{ EDTA negative control}) / (OD_{415} \text{ saline positive control} - OD_{415} \text{ EDTA}) * 100]$ (Table 17A and Table 17B). IC₅₀s were calculated by nonlinear regression (Prism 9, log(inhibitor,bv) vs. response-4 parameter variable slope model) (Table 18A and Table 18B).

Table 17A: Inhibition in Standard AP RBC Hemolysis

Test Article	Engineered Protease	LNP023	Concentration (mM)
% Hemolysis	Not tested	0	10
	3.88	0	3

Test Article	Engineered Protease	LNP023	Concentration (mM)
	47.86	1.16	1
	88.23	0	0.33
	102.20	6.08	0.11
	Not tested	93.92	0.011

Table 17B: Inhibition in Enhanced AP RBC Hemolysis

Test Article	Engineered Protease	LNP023	Concentration (mM)
% Hemolysis	Not tested	0	10
	0	33.94	3
	4.09	108.48	1
	30.45	102.50	0.33
	103.94	110.53	0.11
	104.70	Not tested	0.037
	112.73	Not tested	0.012
	Not tested	102.27	0.011
	99.24	Not tested	0.004
	104.47	Not tested	0.001

Table 18A: Inhibition in Standard AP RBC Hemolysis

Test Article	Engineered Protease	LNP023
IC ₅₀ (mM)	0.92	0.027

Table 18B: Inhibition in Enhanced AP RBC Hemolysis

Test Article	Engineered Protease	LNP023
IC ₅₀ (mM)	0.27	2.6

Example 9: Lung Function Measured in a Mouse Model of Acute Respiratory Distress Syndrome

[0370] FIG. 13 is a schematic depicting the general method for measuring lung function in a mouse model of acute respiratory distress syndrome (ARDS) treated with a chymase-based engineered protease of the disclosure. All references in this Example to engineered proteases are depicted in chymotrypsin numbering. The chymotrypsin numbering key for the modified protease domains of engineered proteases are found in Tables 2, 4, and 6 for uPA, MTSP-1, and chymase, respectively. Briefly, mice received injections of a chymase-based engineered protease C22S/P38Q/K40A/F41R/L99H/V138I/F173Y/D175N/A190S/V213A/S218I/A226R or oral gavage of LNP023 (AdooQ Biosciences A18905), and lung function was measured pre-treatment and 24 hours after LPS instillation.

[0371] After an adaptation period, each animal was weighed and randomly assigned to a treatment group based on body weight. On day 0 (0 hours), mice were anaesthetized and received a single intratracheal instillation (IT) of Lipopolysaccharide (LPS, Sigma) at a dosage of 50 µg per mouse. All animals were monitored for general health status and body weight over the disease course. Respiratory functions were measured by whole-body plethysmography (WBP) on conscious mice at 0 and 24 hours post-LPS IT. Immediately prior to LPS IT, mice received an intravenous (IV) injection of the chymase-based engineered protease listed above at 5.15 mg/kg. Negative control animals received an IV injection of vehicle (PBS). Active comparator animals received a 30 mg/kg dose of LNP023 administered orally. Mice were sacrificed at 24 post-LPS IT.

[0372] Twenty-four hours post administration of the chymase-based engineered protease listed above or LNP023, plethysmography measurements showed a significant protection to pulmonary congestion, suggesting a protection of lung function with treatment. These results are depicted in FIG. 14. The effects to pulmonary congestion index are shown by the fold-change from baseline of the PenH value. The data shown are mean +/- SEM, and **p values are < 0.01 using one-way ANOVA with Dunnett's multiple comparisons test.

[0373] Table 19 shows a comparison of the dose levels of the chymase-based engineered protease of the example and LNP023 dose levels administered *in vivo*

Table 19

Treatment	Dose (mg/kg)	Approximate Molecular Weight (g/mol)	Molar Dose (μmol/kg)	Fold-difference molar dose (LNP023: Engineered Protease)
Engineered Protease	5.15	25100	0.2	~355
LNP023	30	422.5	7.1	

[0374] Table 19 combined with the protective effect of treatment on pulmonary congestion index observed in FIG. 14 demonstrates that the chymase-based engineered protease tested in this example is as efficacious in protecting respiratory function in a mouse model of ARDS as the active comparator LNP023 when administered at approximately a 355-fold lower molar concentration.

[0375] The results suggest the efficient regulation of engineered proteases at low concentrations, while small molecule therapeutics require higher concentrations and frequent dosing.

Example 10: Expression And Purification Of Untagged Chymase-Based Engineered Proteases In a Mammalian Expression System

[0376] HEK293 cells were transiently transfected with chymase-based engineered protease expression vectors, harvested and clarified by depth filtration. All references in this Example to engineered proteases are depicted in chymotrypsin numbering. The chymotrypsin numbering key for the modified protease domains of engineered proteases are found in Tables 2, 4, and 6 for uPA, MTSP-1, and chymase, respectively. The culture harvest is diluted 1.5 fold with 25 mM Tris HCl, pH7.5 (CCS). The CCS is loaded onto a cation exchange column (Capto SP ImPres or similar) at 20 mL/min. The column is washed with 10 CV of 90% Buffer A (25 mM Tris HCL, pH7.5) + 10% Buffer B (with 25 mM Tris HCL, 1M NaCl pH7.5) at 20 mL/min. The recombinant engineered chymase is eluted from the column with a 40 CV linear gradient from

10% Buffer B to 65% Buffer B at 10 mL/min, 5 mL peak fractions containing the engineered chymase are collected, pooled and quantitated by absorbance at 280 nm.

[0377] Chymase-based engineered proteases were activated by incubation with enterokinase following adjustment of the pooled fractions to 150 mM NaCl with Buffer A, and addition of CaCl_2 to 4 mM. Activation was initiated with the addition of enterokinase (EKmax, Invitrogen) and incubated at 37°C overnight. Engineered chymase proteases were approximately 90% activated by this method and were further purified from unactivated chymase and the enterokinase by cation-exchange chromatography using the same procedure outlined above. Pooled fractions are formulated in PBS, 0.1% PS80 (pH 7.4) with purity greater than 98% monomer, HMWS and LMWS less than 2% (FIG. 15).

[0378] FIG. 15 is a SDS-PAGE (reduced) depicting the expression, purification, and activation of a chymase-based engineered protease of the disclosure.

Example 11: Half-Life Extension (HE) and Manufacturability Strategies Using HSA or the IgG1 Fc domain (Fc)

[0379] Nineteen chymase-based engineered proteases based on cleavage activity (Table 10) were transiently expression tested as zymogens in HEK293 cells. All references in this Example to engineered proteases are depicted in chymotrypsin numbering. The chymotrypsin numbering key for the modified protease domains of engineered proteases are found in Tables 2, 4, and 6 for uPA, MTSP-1, and chymase, respectively. Eight of the nineteen expressed as evaluated by SDS-PAGE analysis of clarified tissue culture supernatants (Table 20, Fig. 16). Human HSA or Fc were selected as C-terminal fusion partners for selected variants to express transiently in CHO-S or HEK293 cells with the aim of extending solubility, chemical, and *in vivo* half-life. Both HSA and Fc tagged fusion proteins expressed using the HEK293 transient expression system (FIG. 17). HSA fusion rescued expression for the engineered protease Mutation String Number 1 (MS No. 1), which ranked highly based in specific activity in the original *E. coli*-produced screen.

Table 20: Factor B Cleavage Activity of Exemplary Chymase-Based Engineered Proteases

MS No.	Mutation String	EC50 Fb Cleavage	Expressed in HEK293
1	C22S/P38Q/K40M/F41R/V138I/F173Y/D175R/A190S/V213A/S218V/A226R	28.1	
2	C22S/P38Q/K40L/F41R/V138I/F173Y/D175N/A190S/V213A/S218V/A226R	36.3	Y
3	C22S/P38Q/K40M/F41H/V138I/F173Y/D175R/A190S/V213A/S218V/A226R	47.6	
4	C22S/P38Q/K40M/F41R/V138I/F173L/D175R/A190S/V213A/S218T/A226R	51.0	
5	C22S/P38Q/K40L/F41R/L99Y/V138I/F173Y/D175N/A190S/V213A/S218V/A226R	57.0	Y
6	C22S/P38Q/K40L/F41R/L99N/V138I/F173Y/D175T/A190S/V213A/S218V/A226R	57.6	
7	C22S/P38Q/K40L/F41R/L99H/V138I/F173Y/D175S/A190S/V213A/S218I/A226R	60.3	Y
8	C22S/K40L/F41K/L99N/F173Y/D175N/S218L/A226R	61.0	
9	C22S/P38Q/K40L/F41R/L99N/V138I/F173Y/D175N/A190S/V213A/S218V/A226R	65.3	Y
10	C22S/P38Q/K40A/F41R/L99H/V138I/F173Y/D175N/A190S/V213A/S218V/A226R	71.8	Y
11	C22S/F41K/L99N/F173Y/D175N/S218L/A226R	77.0	
12	C22S/K40L/F41K/F173Y/D175N/S218L/A226R	77.0	
13	C22S/P38Q/K40M/F41R/L99N/V138I/F173Y/D175T/A190S/V213A/S218V/A226R	92.1	Y
14	C22S/P38Q/K40A/F41R/L99H/V138I/F173Y/D175N/A190S/V213A/S218I/A226R	92.9	Y
15	C22S/K40L/F41R/L99N/F173Y/D175N/S218L/A226R	119	
16	C22S/K40L/F41H/L99N/F173Y/D175N/S218L/A226R	139	
17	C22S/P38Q/K40A/F41K/L99H/V138I/D175S/A190S/V213A/S218V/A226R	142.3	Y
18	C22S/F173Y/D175N/A226R	170	
19	C22S/K40L/F41R/L99H/F173Y/D175Y/S218I/A226R		

CLAIMS

1. An engineered protease comprising a modified chymase protease domain, a modified membrane type serine protease 1 (MTSP-1) protease domain, a modified urokinase-type plasminogen activator (uPA) protease domain, or a modified Kallikrein-related peptidase 5 (KLK5) protease domain, wherein the engineered protease is capable of cleaving Factor B.
2. The engineered protease of claim 1, wherein cleavage of Factor B by the engineered protease generates one or more functionally inactive fragments.
3. The engineered protease of claim 2, wherein the one or more functionally inactive fragments are capable of reducing complement activation.
4. The engineered protease of any one of claims 1-3, wherein cleavage of Factor B results in the generation of a Factor B fragment that is reduced in function.
5. The engineered protease of any one of claims 1-4, wherein the Factor B is a non-human primate Factor B.
6. The engineered protease of claim 5, wherein the non-human primate is cynomolgus monkey.
7. The engineered protease of any one of claims 1-4, wherein the Factor B is human Factor B.
8. The engineered protease of claim 7, wherein the Factor B comprises the amino acid sequence as set forth in SEQ ID NO: 1.
9. The engineered protease of any one of claims 1-8, wherein cleavage of Factor B occurs at a site not targeted by Factor D.
10. The engineered protease of claim 9, wherein cleavage at the site not targeted by Factor D generates at least two fragments that are not Ba and Bb.
11. The engineered protease of any one of claims 1-10, wherein cleavage of Factor B results in a reduction of the generation of Factor B cleavage products Ba and Bb as compared to cleavage by Factor D.

12. The engineered protease of any one of claims 1-8, wherein cleavage of Factor B occurs at a site that is targeted by Factor D.
13. The engineered protease of claim 12, wherein the Factor B cleavage site targeted by Factor D comprises QQKR/KIV (SEQ ID NO: 9).
14. The engineered protease of claim 9, wherein the Factor B cleavage site comprises a sequence selected from: WEHR/KGT (SEQ ID NO: 10), KNQKR/QKQ (SEQ ID NO: 11), DVFY/QMI (SEQ ID NO: 12), EGVD/AE (SEQ ID NO: 13), DHKL/KSG (SEQ ID NO: 14), TPW/SLA (SEQ ID NO: 15), KVSEAD (SEQ ID NO: 20), IRPSKG (SEQ ID NO: 4), GGEKRD (SEQ ID NO: 5), GKKEAG (SEQ ID NO: 3), and DHKL/KSG (SEQ ID NO: 21).
15. The engineered protease of claim 9, wherein the Factor B cleavage site comprises a sequence selected from WEHR/KGT (SEQ ID NO: 10) and KNQKR/QKQ (SEQ ID NO: 11), and wherein the engineered protease comprises a modified MTSP-1 protease domain or a modified uPA protease domain.
16. The engineered protease of any one of claims 1-15, wherein the engineered protease comprises a modified MTSP-1 protease domain.
17. The engineered protease of any one of claims 1-15, wherein the engineered protease does not comprise a modified MTSP-1 protease domain.
18. The engineered protease of claim 16, comprising one or more modifications with respect to a MTSP-1 protease domain comprising an amino acid sequence as set forth in SEQ ID NO: 7.
19. The engineered protease of claim 18, wherein the modification is one or more of a substitution, an addition, and deletion of one or more amino acid residues.
20. The engineered protease of claim 16, wherein the one or more modifications is at one or more positions corresponding to one or more positions selected from D622, I640, L678, A686, F703, D705, F706, T707, F708, K719, C731, D734, Y755, Q783, V791, Q802, A814, D828, and K835 in a MTSP-1 protease domain comprising the sequence of amino acids set forth in SEQ ID NO: 18.

21. The engineered protease of claim 16, wherein the one or more modifications are selected from those presented in Table 5A.
22. The engineered protease of claim 16, wherein the one or more modifications are selected from those exemplary mutation strings presented in Table 5B.
23. The engineered protease of any one of claims 1-15, wherein the engineered protease comprises a modified uPA protease domain.
24. The engineered protease of any one of claims 1-15, wherein the engineered protease does not comprise a modified uPA protease domain.
25. The engineered protease of claim 23, comprising one or more modifications with respect to a uPA protease domain comprising an amino acid sequence as set forth in SEQ ID NO: 8.
26. The engineered protease of claim 25, wherein the modification is one or more of a substitution, an addition, and deletion of one or more amino acid residues.
27. The engineered protease of claim 23, wherein the one or more modifications is at one or more positions corresponding to one or more positions selected from S37, D60, L97, G161, R179, H180, V185, Y187, I207, A247, D248, A251, H252, C279, Y308, V316, A343, Q353, R378, K385 in a uPA protease domain comprising the sequence of amino acids set forth SEQ ID NO: 8.
28. The engineered protease of claim 23, wherein the one or more modifications are selected from those presented in Table 3A.
29. The engineered protease of claim 23, wherein the one or more modifications are selected from those exemplary mutation strings presented in Table 3B.
30. The engineered protease of any one of claims 1-19, wherein the engineered protease comprises a modified chymase protease domain.
31. The engineered protease of any one of claims 1-19, wherein the engineered protease does not comprise a modified chymase protease domain.

- 32.** The engineered protease of claim 30, wherein the engineered protease comprises a modified chymase protease domain, and the cleavage site comprises a sequence selected from DVFY/QMI (SEQ ID NO: 12), EGVD/AE (SEQ ID NO: 13), DHKL/KSG (SEQ ID NO: 14), and TPW/SLA (SEQ ID NO: 15).
- 33.** The engineered protease of claim 30, comprising one or more modifications with respect to a chymase protease domain comprising an amino acid sequence as set forth in SEQ ID NO: 6.
- 34.** The engineered protease of claim 33, wherein the modification is one or more of a substitution, an addition, and deletion of one or more amino acid residues.
- 35.** The engineered protease of claim 30, wherein the one or more modifications is one or more positions corresponding to one or more positions selected from S36 C7, V21, P26, G31, R37, K74, K80, I90, L101, L103, F110, V125, F157, D159, S176, A177, F178, K179, L186, V196, G199, A203, A207, F209 in a chymase protease domain comprising the sequence of amino acids set forth in SEQ ID NO: 6.
- 36.** The engineered protease of claim 30, wherein the one or more modifications are selected from those presented in Table 7A.
- 37.** The engineered protease of claim 30, wherein the one or more modifications are selected from those exemplary mutation strings presented in Table 7B.
- 38.** The engineered protease of any one of claims 1-19, wherein the engineered protease comprises a modified KLK5 protease domain, optionally comprising one or more amino acid modifications of SEQ ID NO: 23.
- 39.** The engineered protease of any one of claims 1-19, wherein the engineered protease does not comprise a modified KLK5 protease domain.
- 40.** The engineered protease of any one of claims 1-39, wherein the engineered protease has a k_{cat}/K_m of about 100, about 200, about 300, about 400, about 500, about 600, about 700, about 800, about 900, about 1,000, about 1,000, about 1,100, about 1,200, about 1,300, about 1,400, about 1,500, about 1,600, about 1,700, about 1,800, or about 1,900 $M^{-1} s^{-1}$ for Factor B cleavage.

41. The engineered protease of any one of claims 1-40, wherein the engineered protease has a k_{cat}/K_m of about 10^3 to about $10^9 \text{ M}^{-1} \text{ s}^{-1}$ for Factor B cleavage.
42. The engineered protease of any one of claims 1-41, wherein the engineered protease has an EC_{50} for Factor B of less than about 20 nM.
43. The engineered protease of any one of claims 1-42, wherein the engineered protease has an EC_{50} for Factor B of less than about 1 nM.
44. The engineered protease of any one of claims 1-41, wherein the engineered protease has an EC_{50} for Factor B of about 20, about 25, or about 60 nM.
45. The engineered protease of any one of claims 1-41, wherein the engineered protease has an EC_{50} for cleaving Factor B of about 1,000 to about 4,500 nM.
46. The engineered protease of any one of claims 1-45, wherein the engineered protease has a plasma half-life in human plasma of over about 72 hours.
47. The engineered protease of any one of claims 1-46, wherein the engineered protease has a plasma half-life in human plasma of over about 120 hours.
48. The engineered protease of any one of claims 1-47, wherein the engineered protease has a plasma half-life in human plasma of about 7 days.
49. The engineered protease of claim 48, wherein the catalytic activity is about 10% to about 50%, or about 90% to about 100%.
50. The engineered protease of claim 16, wherein the engineered protease has an increased half-life compared to protease comprising a MTSP-1 protease domain that is not modified.
51. The engineered protease of claim 16, wherein the engineered protease has an increased bioavailability compared to a protease comprising a MTSP-1 protease domain that is not modified.
52. The engineered protease of claim 23, wherein the engineered protease has an increased half-life compared to a protease comprising a uPA protease domain that is not modified.

- 53.** The engineered protease of claim 23, wherein the engineered protease has an increased bioavailability compared to a protease comprising a uPA protease domain that is not modified.
- 54.** The engineered protease of claim 30, wherein the engineered protease has an increased half-life compared to protease comprising a chymase protease domain that is not modified.
- 55.** The engineered protease of claim 30, wherein the engineered protease has an increased bioavailability compared to protease comprising a chymase protease domain that is not modified.
- 56.** The engineered protease of any one of claims 1-55, wherein the engineered protease is non-immunogenic.
- 57.** The engineered protease of any one of claims 1-56, wherein the engineered protease is in a zymogen form.
- 58.** The engineered protease of any one of claims 1-56, wherein the engineered protease is in an active form.
- 59.** The engineered protease of any one of claims 1-58, wherein the engineered protease is fused to a component that extends the half-life of the engineered protease.
- 60.** The engineered protease of claim 59, wherein the component is a Fc domain.
- 61.** The engineered protease of claim 59, wherein the component is a human serum albumin.
- 62.** The engineered protease of any one of claims 1-15, comprising a modified chymase protease domain having at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 95% sequence identity to SEQ ID NO: 6.
- 63.** The engineered protease of claim 62, wherein the modified chymase protease domain of SEQ ID NO: 6 comprises one of the mutation strings of Table 7B.
- 64.** The engineered protease of any one of claims 1-15, comprising a modified membrane type serine protease 1 (MTSP-1) protease domain having at least 50%, at least 55%, at least

60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 95% sequence identity to SEQ ID NO: 7.

65. The engineered protease of claim 64, wherein the modified MTSP-1 protease domain of SEQ ID NO: 7 comprises one of the mutation strings of Table 5B.

66. The engineered protease of any one of claims 1-15, comprising a modified urokinase-type plasminogen activator (uPA) protease domain having at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 95% sequence identity to SEQ ID NO: 22.

67. The engineered protease of claim 66, wherein the modified uPA protease domain of SEQ ID NO: 22 comprises one of the mutation strings of Table 3B.

68. The engineered protease of any one of claims 1-15, comprising a modified Kallikrein-related peptidase 5 (KLK5) protease domain having at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 95% sequence identity to SEQ ID NO: 23.

69. A method of inactivating Factor B, comprising contacting the Factor B with any of the engineered proteases of claims 1-68.

70. A method of treating a disease or condition in a subject in need thereof, comprising administering to the subject any one of the engineered proteases of claims 1-68.

71. The method of claim 70, wherein the disease or condition is associated with dysregulated complement.

72. The method of any one of claims 70-71, wherein the disease or condition is an inflammatory disease or condition.

73. The method of any one of claims 70-72, wherein the treatment is a replacement therapy.

74. The method of any one of claims 70-73, wherein the treatment blocks complement activation.
75. The method of any one of claims 70-74, wherein the treatment modulates autoimmunity.
76. The method of any one of claims 70-75, wherein the disease or condition is a congenital complement deficiency.
77. The method of any one of claims 70-76, wherein the treatment is for endothelial or kidney cell injury.
78. The method of any one of claims 70-77, wherein the disease or condition is selected from lupus nephritis, C3 glomerulopathy (C3G), primary IgA nephropathy, kidney transplant ischemia and reperfusion (I/R) injury, antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV), sepsis, acute respiratory distress syndrome (ARDS), SARS-associated coronavirus (SARS-CoV), atypical hemolytic uremic syndrome (aHUS), membranous nephropathy (MN) and paroxysmal nocturnal hemoglobinuria (PNH).
79. The method of any one of claims 70-78, wherein the disease or condition is a control protein deficiency.
80. The method of any one of claims 70-78, wherein the disease or condition is a secondary complement disorder.
81. The method of any one of claims 70-78, wherein the disease or condition is an immunity-related disease or condition.
82. The method of any one of claims 70-81, wherein the engineered protease is administered to the subject subcutaneously.
83. The method of claim 82, wherein the engineered protease is activated in situ at the site of a dysregulated complement component.
84. The method of any of any one of claims 70-83, wherein the engineered protease is provided in a liquid stable formulation.

- 85.** A pharmaceutical composition comprising any of the engineered proteases of claims 1-68, and optionally a pharmaceutically acceptable carrier.
- 86.** The pharmaceutical composition of claim 85, wherein the engineered protease is provided in a liquid stable formulation.
- 87.** The pharmaceutical composition of any one of claims 85-86, wherein the composition is formulated for subcutaneous administration.