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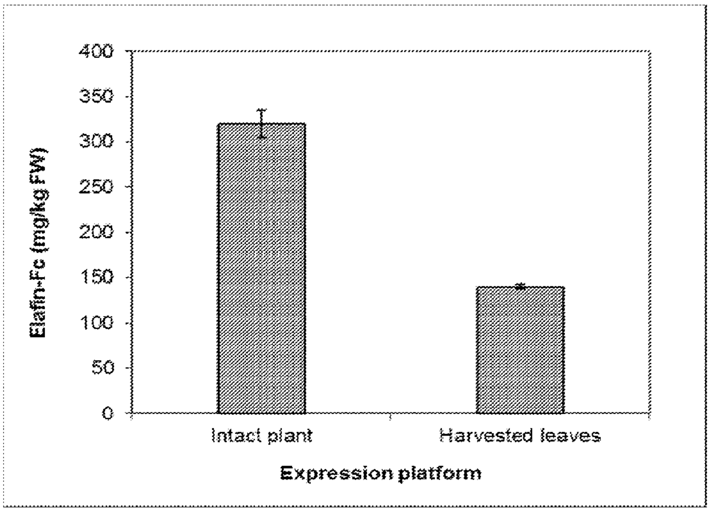

FIG. 5A

(57) Abstract: The present invention provides plant-made elafin-Fc fusion proteins for treating inflammatory diseases, e.g., inflammatory lung diseases. In certain embodiments, the fusion proteins comprise one or more point mutations, which confer improved properties, such as increased resistance to oxidation, cleavage, and increased half-life. The present invention additionally provides polynucleotides encoding the fusion proteins, recombinant cells and expression vectors, and transgenic plants comprising the fusion protein coding sequences. The present invention further provides methods for the production of the fusion proteins.

WO 2017/192580 A1

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NOVEL FUSION PROTEINS FOR TREATING INFLAMMATORY DISEASES

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application No. 62/330,717, filed May 2, 2016, the disclosure of which is hereby incorporated by reference in its entirety for all purposes.

REFERENCE TO A SEQUENCE LISTING

[0002] The Sequence Listing written in file SequenceListing_1046120.txt created on May 1, 2017, 21,225 bytes, machine format IBM-PC, MS-Windows operating system, is hereby incorporated by reference in its entirety for all purposes.

BACKGROUND OF THE INVENTION

[0003] Lung diseases affect a large population in the United States every year and account for the leading causes of death. Severe alpha-1 antitrypsin deficiency (AATD) is an inherited disorder that may cause lung disease. People with AATD usually develop shortness of breath following mild activity, reduced ability to exercise, and wheezing. Affected individuals often develop emphysema, which is characterized by difficulty in breathing, a hacking cough, and a barrel-shaped chest. Cystic fibrosis (CF) is another genetic disease that affects mostly the lungs, and patients having CF often show difficulty breathing and coughing up mucus as a result of frequent lung infections. Other signs and symptoms include sinus infections, poor growth, fatty stool, clubbing of the fingers and toes, and infertility in males. Both AATD and CF have approximately the same, 1:2500 overall incidence rate in Caucasians.

[0004] Currently, the only commercially available intravenous (IV) replacement therapies for humans with AATD or CF are derived from pooled, donated human plasma, and the costs for the infusion products are very high – between $127,000 and $146,000 per year exclusive of infusion costs – and somewhat less for inhalation therapy. Patients with AATD-related emphysema need replacement therapy of 60 mg/kg of weekly alpha-1 antitrypsin (AAT) IV infusions (~200 g/year/patient). These infusion products include Prolastin-C® from Grifols, Aralast NP™ from Baxalta, Zemaira® from CSL Behring and Glassia® from Kamada Ltd via Baxalta. Although these products are safe and effective, the products differ in purity, molecular heterogeneity, and supply is potentially constrained by plasma availability and requires complex and expensive collection, purification, and sterilization and preservation/distribution challenges. Importantly, supply and high costs limit AAT's availability to patients requiring the therapy under present replacement therapy guidelines [4-7]. Plasma-derived AAT is also sensitive to oxidation of methionine at position 358, which causes loss of anti-elastase activity required to be effective in treating AATD [8]. Although recombinant AAT has been produced in *E. coli*, insect, yeast, mammalian and plant expression systems, the recombinant protein has limited biological activity, low expression, high production cost and/or incorrect glycosylation that can impair both biological activity and pharmacokinetics [9-11].

[0005] As such, there is a need in the art for new therapies to treat lung diseases including AATD and CF that overcome the drawbacks associated with current replacement therapies. The present disclosure addresses this and other needs.

BRIEF SUMMARY OF THE INVENTION

[0006] In a first aspect, the present disclosure provides a fusion protein comprising a signal peptide, an elafin region, a linker, and a Fc region. In certain embodiments, the elafin region comprises a sequence that is at least 80%, 85%, 90%, 95%, or 98% identical to SEQ ID NO:1. In some embodiments, the fusion protein comprises one or more point mutations in the elafin region relative to SEQ ID NO:1. The point mutations can be one or more of the following mutations in the elafin region of the fusion protein: V5G, V9G, M25L, and M51V. In certain embodiments, the fusion protein comprising the one or more point mutations is more resistant to proteolytic cleavage and/or more resistant to oxidation compared to a control fusion protein without the one or more point mutations in the elafin region.

[0007] In certain embodiments, the fusion protein described above comprises a signal peptide comprising a sequence that is at least 80%, 85%, 90%, 95%, or 98% identical to SEQ ID NO:2. The fusion protein can also comprise a linker sequence that is at least 80%, 85%, 90%, 95%, or 98% identical to SEQ ID NOS:3 or 15.

[0008] In certain embodiments, the fusion protein described above comprises an Fc region comprising a sequence that is at least 80%, 85%, 90%, 95%, or 98% identical to SEQ ID NO:4. The Fc region of the fusion protein can comprise one or more point mutations, *e.g.*, M203L and/or N209S, in the Fc region as set forth in SEQ ID NO:4. In certain embodiments, the fusion protein has a longer half-life compared to a control fusion protein without the one or more point mutations in the Fc region. In some embodiments, the Fc region of the fusion protein is galactosylated and sialylated, *e.g.,* at an N-glycan site such as at amino acid position 72 of SEQ ID NO:4.

[0009] In certain embodiments, the fusion protein comprises a sequence that is at least 80%, 85%, 90%, 95%, or 98% identical to any one of SEQ ID NOS:5-7 and 13.

[0010] In another aspect, the present disclosure provides a polynucleotide encoding the fusion protein described above. In certain embodiments, the sequence of the polynucleotide has been codon optimized for expressing the fusion protein in a plant. In certain embodiments, the polynucleotide is at least 80%, 85%, 90%, 95%, or 98% identical to any one of SEQ ID NOS:8-10 and 14.

[0011] In another aspect, the present disclosure provides an expression vector comprising the polynucleotide described above. In certain embodiments, the expression vector comprises a CaMV 35S promoter. In certain embodiments, the expression vector comprises an Omega leader sequence of SEQ ID NO:16.

[0012] In another aspect, the present disclosure provides a recombinant cell comprising the polynucleotide described above. In certain embodiments, the recombinant cell is a plant cell, *e.g.,* a cell derived from *Nicotiana benthamiana.* In some embodiments, the recombinant cell lacks fucosylation and xylosylation pathways and has galactosylation pathways.

[0013] In another aspect, the present disclosure provides a plant comprising the recombinant cell described above. In certain embodiments, the plant is tobacco.

[0014] In yet another aspect, the present disclosure provides a method for producing a fusion protein comprising: (a) providing a polynucleotide described above, (b) introducing the polynucleotide into a plant tissue, thereby expressing the fusion protein in the plant tissue, and (c) recovering the fusion protein from the plant tissue. In certain embodiments, the plant tissue expresses a viral RNA gene silencing suppressor. In certain embodiments, the polynucleotide is introduced into the plant tissue by *Agrobacterium.* In certain embodiments, the polynucleotide is introduced into the plant tissue by vacuum infiltration. In certain embodiments, the introduction of the polynucleotide into the plant is by combining agrobacterium with the plant tissue using transient agroinfiltration to form a mixture and to allow infection of at least one plant cell of the plant tissue, and wherein the fusion protein is recovered from the plant tissue after a period of time sufficient for the plant tissue to transiently express the fusion protein, *e.g.*, at least 3 days, at least 4 days, at least 5 days, or at least 6 days.

[0015] In yet another aspect, the present disclosure provides a composition comprising the fusion protein described above.

[0016] In yet another aspect, the present disclosure provides a method for treating an inflammatory disease in a subject, the method comprising administering to the subject an effective amount of the composition described above. In certain embodiments, the inflammatory disease is selected from the group consisting of an inflammatory pulmonary disease, inflammatory vascular disease, inflammatory systemic disease, and inflammation triggered by reperfusion injury. In certain embodiments, the inflammatory pulmonary disease is selected from the group consisting of alpha-1 antitrypsin deficiency (AATD), cystic fibrosis (CF), and chronic obstructive pulmonary disease (COPD).

[0017] Other objects, features, and advantages of the present invention will be apparent to one of skill in the art from the following detailed description and figures.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] FIG. 1 illustrates the various functional regions of an elafin-Fc fusion protein (corresponding to variant 4 (SEQ ID NO:7)): the signal peptide; the elafin region; the linker; and the Fc region. The elafin region comprises the following point mutations relative to the native elafin: V5G, V9G, M25L, and M51V. The Fc region comprises the following point mutations relative to the native Fc: M203L and N209S.

[0019] FIG. 2 illustrates the various functional regions of another elafin-Fc fusion protein (corresponding to variant 5 (SEQ ID NO:13)): Variant 5 is identical to variant 4 except for the linker region having a sequence of SEQ ID NO:15 instead of SEQ ID NO:3.

[0020] FIG. 3 is a schematic representation of a binary plasmid (pDP16.0707) encoding the elafin-Fc fusion protein corresponding to SEQ ID NO:13.

[0021] FIG. 4 shows the results of Dot blot experiments performed to detect the elafin-Fc fusion protein corresponding to SEQ ID NO:13 in plant extracts.

[0022] FIG. 5A shows the expression of the elafin-Fc fusion protein corresponding to SEQ ID NO:13 detected by ELISA (FIG. 5A) and Western blot (FIG. 5B). Extracts from intact plant vs. harvested leaves at 5 days of post infiltration were analyzed in ELISA and Western blot. 10 µl of the crude extracts from each sample was analyzed by Western blot.

[0023] FIG. 6A shows a chromatogram of the proteins extracted from intact plant. FIG. 6B shows the elution of the elafin-Fc fusion protein corresponding to SEQ ID NO:13. FIG. 6C shows an SDS-PAGE analysis of the elafin-Fc fusion protein corresponding to SEQ ID NO:13.

[0024] FIG. 7A shows a chromatogram of the proteins extracted from intact plant. FIG. 7B shows the elution of the elafin-Fc fusion protein corresponding to SEQ ID NO:13. FIG. 7C shows an SDS-PAGE analysis of the elafin-Fc fusion protein corresponding to SEQ ID NO:13.

DETAILED DESCRIPTION OF THE INVENTION

I. INTRODUCTION

[0025] The present invention provides an elafin-Fc fusion protein for treating inflammatory diseases, especially inflammatory lung diseases, and methods for producing the fusion protein. The fusion protein can be further engineered to be oxidation- and proteolytic-cleavage resistant, as well as having an increased half-life compared to the native human elafin. The fusion protein can also be produced by transient expression in plants, which is rapid (*e.g.,* maximum production of 4-6 days post-agroinfiltration), scalable (*e.g.,* commercial facilities are capable of producing 240 kg/year of purified protein, which is enough to treat 1,200 AATD patients per year), and cost effective. In addition, the plant-produced elafin-Fc fusion protein is oxidation and proteolytic cleavage-resistant, which ensures that the fusion protein has an increased half-life compared to the native human elafin. The addition of IgG Fc at the C-terminus allows efficient and scalable purification of the elafin-Fc fusion protein using standard protein A chromatography. This novel chimeric protein can be produced transiently in a *Nicotiana benthamiana* plant host, providing a scalable, cost-effective approach that enables reductions in both capital investments and cost-of-goods sold (>50%) compared with traditional mammalian cell cultures [73].

II. DEFINITIONS

[0026] The terms “a,” “an,” or “the” as used herein not only include aspects with one member, but also include aspects with more than one member. For instance, the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and reference to "the agent" includes reference to one or more agents known to those skilled in the art, and so forth.

[0027] The terms "subject", "patient" or "individual" are used herein interchangeably to refer to a human or animal. For example, the animal subject may be a mammal, a primate (*e.g.*, a monkey), a livestock animal (*e.g.*, a horse, a cow, a sheep, a pig, or a goat), a companion animal (*e.g.*, a dog, a cat), a laboratory test animal (*e.g.*, a mouse, a rat, a guinea pig, a bird), an animal of veterinary significance, or an animal of economic significance.

[0028] The term "nucleic acid" or "polynucleotide" includes deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions), alleles, orthologs, SNPs, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.* 19:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.* 260:2605-2608 (1985); and Cassol *et al.* (1992); Rossolini *et al.*, *Mol. Cell. Probes* 8:91-98 (1994)). The term nucleic acid is used interchangeably with gene, cDNA, and mRNA encoded by a gene.

[0029] The terms "polypeptide," "peptide," and "protein" are used interchangeably herein to include a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymers. As used herein, the terms encompass amino acid chains of any length, including full-length proteins (*i.e.*, antigens), wherein the amino acid residues are linked by covalent peptide bonds.

[0030] The term "amino acid" includes naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline, γ-carboxyglutamate, and O-phosphoserine. Amino acid analogs include compounds that have the same basic chemical structure as a naturally occurring amino acid, *i.e.*, an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. "Amino acid mimetics" include chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid.

[0031] Amino acids may be referred to herein by either the commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

[0032] As to amino acid sequences, one of skill will recognize that individual substitutions, deletions and/or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and/or alleles.

[0033] The following eight groups each contain amino acids that are conservative substitutions for one another:

1) Alanine (A), Glycine (G);
2) Aspartic acid (D), Glutamic acid (E);
3) Asparagine (N), Glutamine (Q);
4) Arginine (R), Lysine (K);
5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V);
6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W);
7) Serine (S), Threonine (T); and
8) Cysteine (C), Methionine (M)

(*see, e.g.*, Creighton, *Proteins* (1984)).

[0034] The term "fusion protein" commonly refers to a protein that comprises two or more subsequences that are not found in the same relationship to each other in nature. For purposes of this disclosure, the fusion protein refers to the elafin-Fc fusion protein, which comprises a signal peptide, an elafin region, a linker, and a Fc region.

[0035] The term "recombinant" when used with reference, *e.g.*, to a cell, or nucleic acid, protein, or vector, indicates that the cell, nucleic acid, protein or vector, has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. For example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under-expressed, or not expressed at all.

[0036] The term "expression vector" includes a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular nucleic acid in a host cell. The expression vector can be part of a plasmid, virus, or nucleic acid fragment. Typically, the expression vector includes a nucleic acid to be transcribed operably linked to a promoter.

[0037] The term "therapeutically effective amount" or "effective mount" includes an amount or quantity effective, at dosages and for periods of time necessary, to achieve the desired therapeutic or prophylactic result.

[0038] The term "elafin" as used herein encompasses the polypeptide comprising the sequence of SEQ ID NO:1 as well as homologues, derivatives or fragments thereof that have significant sequence identity with SEQ ID NO:1 and retains the inhibitory activity against an elastase, *e.g.*, a human neutrophil elastase or porcine pancreatic elastase.

[0039] The term "plant" includes whole plants, shoot vegetative organs/structures (*e.g.*, leaves, stems and tubers), roots, flowers and floral organs/structures (*e.g.*, bracts, sepals, petals, stamens, carpels, anthers and ovules), seed (including embryo, endosperm, and seed coat) and fruit (the mature ovary), plant tissue (*e.g.*, vascular tissue, ground tissue, and the like) and cells (*e.g.*, guard cells, egg cells, and the like), and progeny of same.

[0040] The term "administering" includes oral administration, topical contact, administration as a suppository, intravenous, intraperitoneal, intramuscular, intralesional, intrathecal, intranasal, or subcutaneous administration, or the implantation of a slow-release device, *e.g.*, a mini-osmotic pump, to a subject. Administration is by any route, including parenteral and transmucosal (*e.g.*, buccal, sublingual, palatal, gingival, nasal, vaginal, rectal, or transdermal). Parenteral administration includes, *e.g.*, intravenous, intramuscular, intra-arteriole, intradermal, subcutaneous, intraperitoneal, intraventricular, and intracranial. Other modes of delivery include, but are not limited to, the use of liposomal formulations, intravenous infusion, transdermal patches, *etc.* One skilled in the art will know of additional methods for administering a therapeutically effective amount of a fusion protein described herein.

III. DESCRIPTION OF THE EMBODIMENTS

A. Components of the elafin-Fc fusion protein

[0041] Native human elafin (SEQ ID NO:1) is a soluble protein with 57 amino acids and a molecular weight of about 6 kDa [17]. Native elafin is expressed predominantly in epithelial tissue and potently inhibits the neutrophil-derived serine proteases (elastase) by a competitive tight-binding mechanism. Native elafin is prone to oxidation at a methionine 25 residue which makes the protein unable to efficiently inhibit elastase activity [15]. In addition, native elafin is cleaved by its cognate enzyme neutrophil elastase in sputum from individuals with cystic fibrosis which will lower the treatment efficacy [16]. Moreover, the half-life of native elafin in plasma is only 115 minutes on average [17].

[0042] The elafin-Fc fusion protein described herein comprises a signal peptide, an elafin region, a linker, and a Fc region. *See, e.g.*, FIGS. 1 and 2. The elafin-Fc fusion protein of the invention comprises an elafin region that may comprise one or more mutations relative to the native elafin. Preferably, the elafin region comprises a sequence that is at least 80%, 85%, 90%, 95%, or 98% identical to the native elafin, SEQ ID NO:1. Generally, any amino acid from the sequence depicted in SEQ ID NO:1 can be deleted and substituted with another amino acid as long as the elafin protein's inhibitory activity on the neutrophil-derived serine proteases, *e.g.*, elastases, is not lost. In some cases, the mutations are introduced via one or more conservative substitutions in order to retain such inhibitory activity.

[0043] The elafin region of the fusion protein may comprise mutations that can confer improved properties to the fusion protein, *e.g.*, greater resistance to oxidation and/or greater resistance to proteolytic cleavage as compared to a control fusion protein without the mutations. Mutations that confer greater resistance to proteolytic cleavage to the fusion protein include, for example, the V→G substitution at position 5 ("V5G") of SEQ ID NO:1

or at position 9 ("V9G") of SEQ ID NO:1 [36]. Mutations that confer increased resistance to oxidation to the fusion protein include, for example, the M→L substitution at position 25 ("M25L") of SEQ ID NO:1 or the M→V substitution at position 51 ("M51V") of SEQ ID NO:1 [40]. The amino acid position 1 is the first amino acid (alanine) in the elafin region (SEQ ID NO:1). The elafin region of the fusion protein can comprise one or more of these point mutations and the fusion protein can be more resistant to proteolytic cleavage and/or more resistant to oxidation as compared to a control fusion protein without the one or more point mutations.

[0044] The signal peptide of the fusion protein can be any signal peptide that can facilitate secretion of the fusion protein from plant cells to the apoplast. In a particular embodiment, the signal peptide is the 25-amino-acid signal peptide from Oryza sativa α-amylase (Ramy3D SP), SEQ ID NO:2. In some embodiments, the signal peptide used in the fusion protein comprises a sequence that is at least 80%, 85%, 90%, 95%, or 98% identical to SEQ ID NO:2.

[0045] The linker region of the fusion protein is a polypeptide sequence that connects the elafin region with a Fc region, which provides flexibility for the Fc fusion and allows elafin-Fc to bind the target, *e.g.*, neutrophil elastase [38]. Any immunoglobulin hinge region linker or a portion thereof can be used as the linker for the fusion protein described herein, for example, those described in U.S. Patent No. 6,165,476. In one embodiment, the linker is the human IgG1 hinge region, which comprises a sequence of SEQ ID NO:3, or a portion thereof. In another embodiment, the linker is the human IgG2 hinge region, which comprises a sequence of SEQ ID NO:15 (*see,* UniProt Accession No. P01859), or a portion thereof. In some cases, the linker comprises a disulfide bond linkage for Fc dimerization, which can beneficially maintain Fc function. In one embodiment, the linker comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, or 98% identical to SEQ ID NO:3 or SEQ ID NO:15.

[0046] The elafin-Fc fusion protein comprises an immunoglobulin Fc domain ("Fc"). The attachment of the Fc domain to elafin significantly increases the protein's plasma half-life, for at least the following reasons. The Fc domain can interact with the salvage neonatal Fc-receptor, resulting in increased protein stability. The Fc domain also increases the size of the protein, resulting in a slower renal clearance. The Fc domain also folds independently and thus improves the solubility and stability of the elafin protein. The Fc domain additionally offers the technological benefits of easy cost-effective purification by protein-G/A affinity chromatography during manufacture.

[0047] In certain embodiments, the Fc region comprises a sequence of SEQ ID NO:4. In some embodiments, the Fc region comprises a sequence that is at least 80%, 85%, 90%, 95%, or 98% identical to SEQ ID NO:4. In some embodiments, the Fc region comprises one or more point mutations that increase the half-life of the fusion protein as compared to the fusion protein without the one or more point mutations in the Fc region. In particular embodiments, the Fc region comprises at least one of the point mutations selected from the group consisting of the M→L substitution at position 203 ("M203L") of SEQ ID NO:4 and the N→S substitution at position 209 ("N209S") of SEQ ID NO:4. In some embodiments, the Fc region of the fusion protein described herein is both galactosylated and sialyated at position 72 of SEQ ID NO:4 to boost the anti-inflammatory effect of the fusion protein [38-39].

[0048] Various mutations that can be introduced into the fusion protein and the properties of the protein comprising these mutations are shown in Table 1.

Table 1. Various mutations in the elafin-Fc fusion protein and the properties thereof

Domain	**Amino acid position(s)**	**Mutation(s)**	**Properties**
Signal peptide (Ramy 3D)	N-terminal	Not mutated	Elafin protein will be targeted to apoplast
Elafin	5 and 9 of SEQ ID NO:1	V5G, V9G (GG variant)	Resistant to proteolytic cleavage [36]
Elafin	25 and 51 of SEQ ID NO:1	M25L, M51V	Resistant to oxidation (U.S. Pat. No. 5,734,014)
Linker	Hinge region of IgG1 Hinge region of IgG2	Not mutated	Hinge region of IgG1 is more flexible for the Fc fusion which will allow the binding of elafin-Fc to elastase (U.S. Pat. No. 6,165,476; U.S. Pat. Pub. No. 2013/0011399; U.S. Pat. No. 8,980,266)
Fc of IgG1	203 and 209 of SEQ ID NO:4	M203L, N209S	Increased half-life (through FcRn binding) [37].

N-glycan of Fc	72 of SEQ ID NO:4	Expressed in xylose fucose free RNAi and GalT + *N.benthamiana*	Galactosylation of Fc enhances the anti-inflammatory effect [38].
N-glycan of Fc	72 of SEQ ID NO:4	Sialylation by 1 step *in vitro* method	Sialylation of Fc also enhances the anti-inflammatory effect [39].

[0049] As compared to existing products, the elafin-Fc fusion proteins of the present invention have superior properties, as shown in Table 2.

Feature	**Present Invention**	**FDA approved plasma AAT**	**Elafin produced from yeast (Proteo Inc.)**
Native or recombinant?	Recombinant	Native	Recombinant
Source	Plant	Human plasma	Yeast (*Pichia*)
Oxidation Resistant?	Yes (Elafin mutant: M25L, M51V)	No (M358) sensitive	No (M25, M51) sensitive
Proteolytic cleavage resistant?	Yes (Elafin mutant: V5G, V9G)	No (Pro357-M358) sensitive	No (V5, V9) sensitive
Serum half-life?	21 days (Fc mutant: M203L and N209S)	5 days	115 minutes

B. Sequence variants

[0050] It will readily be appreciated by those of skill in the art that the instant disclosure includes a variety of polynucleotide sequences that are capable of encoding the various functional regions of the elafin-Fc fusion proteins and have the required function. The polynucleotide sequences may encode polypeptides including those sequences with deletions, insertions, or substitutions of different nucleotides, which result in a polynucleotide encoding a polypeptide with at least one functional characteristic of the instant polypeptides, as described herein. Included within this definition are polymorphisms which may or may not be readily detectable using a particular oligonucleotide probe of the polynucleotide encoding the instant polypeptides, and improper or unexpected hybridization to allelic variants, with a locus other than the normal chromosomal locus for the polynucleotide sequence encoding the instant polypeptides.

[0051] Sequence alterations that do not change the amino acid sequence encoded by the polynucleotide are termed "silent" variations. Due to the degeneracy of the genetic code, with the exception of the codons ATG and TGG, encoding methionine and tryptophan, respectively, any of the possible codons for the same amino acid can be substituted by a variety of techniques, for example, site-directed mutagenesis, available in the art. Accordingly, any and all such variations of a sequence selected from the Sequence Listing are a feature of the instant disclosure.

[0052] In addition to silent variations, other conservative variations that alter one or a few amino acids in the encoded elafin-Fc fusion protein, can be made without altering the function of the polypeptide. For example, substitutions, deletions and insertions introduced into the sequences provided herein are also envisioned. Amino acid substitutions are typically of single residues; insertions usually will be on the order of about from 1 to 10 amino acid residues; and deletions will range about from 1 to 30 residues. In particular embodiments, deletions or insertions are made in adjacent pairs, for example, a deletion of two residues or insertion of two residues. Substitutions, deletions, insertions or any combination thereof can be combined to arrive at a sequence. The mutations that are made in the polynucleotide encoding the various functional regions, *e.g.*, the elafin region and the Fc region, of the elafin-Fc fusion protein should not place the sequence out of the reading frame and should not create complementary regions that could produce secondary mRNA structure. Preferably, the polypeptide encoded by the DNA performs the desired function.

[0053] Conservative substitutions are those in which at least one residue in the amino acid sequence has been removed and a different residue inserted in its place. Such substitutions generally are made when it is desired to maintain the activity of the protein. Various functional regions of the elafin-Fc fusion protein confer specific activities to the fusion protein. For example, the elafin region can confer the activity of inhibiting the neutrophil-derived serine proteases (elastase). Although all conservative amino acid substitutions (*e.g.*, one basic amino acid substituted for another basic amino acid) in a polypeptide will not necessarily result in the polypeptide retaining the same activity as the native polypeptide, it is expected that many of these conservative mutations would result in the polypeptide retaining its activity.

[0054] Sequence variants encoding various functional regions of the elafin-Fc fusion proteins can be produced by modifying the respective wild-type sequences according to methods well-known to the skilled in the art. Such methods include, but not limited to, mutagenesis by PCR, which uses primers designed to contain desired changes [41]; nested primers to mutate a target region [42]; and inverse PCR, which amplifies a region of unknown sequence using primers orientated in the reverse direction [43-44]. Many other mutation and evolution methods are also available and expected to be within the skill of a person of ordinary skill in the relevant art.

[0055] The polynucleotides encoding the elafin-Fc fusion proteins described herein may also be chemically synthesized in accordance with the desired sequence by a known synthesis process. These sequences can be cloned to an expression vector using well-established cloning procedures.

[0056] Chemical or enzymatic alterations of expressed nucleic acids and polypeptides can be performed by standard methods. For example, sequences can be modified by the addition of lipids, sugars, peptides, organic or inorganic compounds, by the inclusion of modified nucleotides or amino acids, or the like. For example, protein modification techniques are illustrated in Ausubel *et al.* [45]. These modification methods can be used to modify any given sequence, or to modify any sequence produced by the various mutation and artificial evolution modification methods noted herein.

[0057] Accordingly, the present invention provides for modification of any given nucleic acid by mutation, chemical or enzymatic modification, or other available methods, as well as for the products produced by practicing such methods, *e.g.,* using the sequences herein as a starting substrate for the various modification approaches.

C. Sequence identity determination

[0058] Sequence identity or similarity may be determined using standard techniques known in the art, including, but not limited to, the local sequence identity algorithm of Smith & Waterman [46]; the sequence identity alignment algorithm of Needieman & Wunsch [47]; the search for similarity method of Pearson & Lipman [48]; the computerized implementations of these algorithms (GAP, BESTFIT, FASTA, BLAST, Clustal Omega, and TFASTA in the Wisconsin Genetics Software Package, Genetics computer Group, 575 Science Drive, Madison, Wl); or the Best Fit sequence program described by Devereux *et al.* [49], preferably using the default settings.

[0059] Sequence identity can also be determined by inspection. For example, the sequence identity between sequence A and sequence B, aligned using the software above or manually, can be determined by dividing the length of sequence A, minus the number of gap residues in sequence A, minus the number of gap residues in sequence B, into the sum of the residue matches between sequence A and sequence B, times one hundred.

D. Codon optimization of the elafin-Fc coding sequence

[0060] Generally, codon optimization can be used to increase the rate of translation or to produce recombinant RNA transcripts having desirable properties, such as a longer half-life, as compared with transcripts produced using a non-optimized sequence. Plant and mammals have different codon preferences, so that mammalian genes frequently use codons which are rarely used in plants. As a result, mammalian genes may be expressed very poorly in plants. The present disclosure provides elafin-Fc fusion protein coding sequences that have been engineered to match the codon usage pattern of the plant host to maximize expression efficiency. Methods for codon optimization are readily available, for example, optimizer, accessible free of charge at http://genomes.urv.es/OPTIMIZER, and GeneGPS® Expression Optimization Technology from DNA 2.0 (Newark, California). In particular embodiments, the coding sequence is codon-optimized for expression in *N. benthamiana* using the OptimumGene^{TM} algorithm from GenScript (Piscataway, New Jersey).

E. Vectors and Promoters

[0061] The expression vectors provided by this disclosure comprise polynucleotides encoding the elafin-Fc fusion proteins. The expression vectors preferably are plant expression vectors. The constructs typically comprise a vector, such as a plasmid, a cosmid, a phage, a virus (*e.g.*, a plant virus), a bacterial artificial chromosome (BAC), a yeast artificial chromosome (YAC), or the like, into which a nucleic acid sequence of the invention has been inserted, in a forward or reverse orientation. In a particular aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available.

[0062] Typically, the expression vectors include the elafin-Fc coding sequence under the transcriptional control of 5' and 3' regulatory sequences. Such plant transformation vectors typically also contain a promoter (*e.g.*, a regulatory region controlling inducible or constitutive, environmentally- or developmentally-regulated, or cell- or tissue-specific expression), a transcription initiation start site, an RNA processing signal (such as intron splice sites), a transcription termination site, and/or a polyadenylation signal.

[0063] Non-limiting examples of constitutive plant promoters which can be useful for expressing the elafin-Fc fusion protein sequence include the cauliflower mosaic virus (CaMV) 35S promoter, which confers constitutive, high-level expression in most plant tissues [50]; the nopaline synthase promoter [51]; the octopine synthase promoter [52]; and the Ramy 3D promoter [64]. A variety of plant gene promoters that regulate gene expression in response to environmental, hormonal, chemical, developmental signals, and in a tissue-active manner can also be used for expression of the elafin-Fc fusion protein.

[0064] The expression vectors described herein can also include RNA processing signals that can be positioned within, upstream or downstream of the coding sequence. In addition, the expression vectors can include additional regulatory sequences from the 3'-untranslated region of plant genes, *e.g.*, a 3' terminator region to increase mRNA stability of the mRNA, such as the PI-II terminator region of potato or the octopine or nopaline synthase 3' terminator regions.

[0065] Specific initiation signals can aid in efficient translation of the elafin-Fc coding sequences. These signals can include, *e.g.*, the ATG initiation codon and adjacent sequences. In cases where a coding sequence, its initiation codon and upstream sequences are inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only coding sequence (*e.g.*, a mature protein coding sequence), or a portion thereof, is inserted, exogenous transcriptional control signals including the ATG initiation codon can be separately provided. The initiation codon is provided in the correct reading frame to facilitate transcription. Exogenous transcriptional elements and initiation codons can be of various origins, both natural and synthetic. The efficiency of expression can be enhanced by the inclusion of enhancers appropriate to the cell system in use.

F. Host plants

[0066] The expression vector comprising an elafin-Fc fusion protein coding sequence can be introduced into a variety of host plant species, *e.g., Nicotiana benthamiana, Nicotiana tobacum, Lactuca sativa, and Solanum lycopersicum.* In particular embodiments, the host plant is *Nicotiana benthamiana.*

[0067] In some embodiments, the host plant used for the invention lacks enzymes responsible for plant-specific glycans, such as xylosyltranferases and fucosyltransferases. Genetic knock-out of xylosyltranferases and fucosyltransferases can be established by homologous recombination [53] or by screening mutant libraries [54], or gene silencing [55]. The plant specific glycosylation, *i.e.*, the addition of xylose and fucose, can also be inhibited via the expression of mutant galactosyltransferases, which appear to result in intermediate galactosylated N-glycan structures that are not the substrates for the xylosyl- and fucosyltransferases [56]. In particular embodiments, the host plants lacking enzymes responsible for plant-specific glycans also have human-like glycosylation pathways. Methods for humanizing glycosylation pathways in plants are well known, for example, by introducing into the host plant human β1,4-galactosyltranferases as described in Palacpac et al. [57].

[0068] In some embodiments, in order to counteract the host plant's RNA-mediated gene silencing, a defense mechanism that inhibits expression of exogenous protein, a viral RNA silencing suppressor is introduced into the host plant [58]. The presence of the viral RNA silencing suppressor is expected to improve the expression efficiency of the elafin-Fc fusion protein. The viral RNA silencing suppressor gene can be introduced prior to, or simultaneously with, the introduction of elafin-Fc gene into the host plant to boost elafin-Fc protein expression level. Non-limiting examples of RNA gene silencing suppressors that can be used in the invention include P19 and Hc-Pro. In particular embodiments, the viral RNA gene silencing suppressor is Tomato bushy stunt virus P19.

G. Agrobacterium mediated transformation

[0069] The elafin-Fc coding sequence can be transformed into host plants to express the fusion protein, stably or transiently, using methods well established in the art. In particular embodiments, the elafin-Fc coding sequence, under the control of a strong promoter, *e.g.*, the CaMV 35S constitutive promoter, is introduced into a host plant via agroinfiltration and is expressed transiently. Methods of agroinfiltration are well known, for example, as disclosed in Susammenfassung [59].

[0070] In some embodiments, the elafin-Fc expression vector is first introduced into the agrobacteria, for example, the EHA105pCH32 strain, using standard methods, such as electroporation or a heat shock method. In certain instances, the transformed agrobacteria are grown in a liquid culture and the resulting bacteria are washed and suspended into a suitable solution to infect the host plant.

[0071] A number of ways can be used to infect the host plant. In one embodiment, the bacteria solution is placed in a syringe, and the tip of the syringe is pressed against the underside of a leaf while simultaneously applying gentle counter pressure to the other side of the leaf. The agrobacterium is then injected into the airspaces inside the leaf through stomata, or sometimes through a tiny incision made to the underside of the leaf. Vacuum infiltration is another way to penetrate *Agrobacterium* deep into plant tissue. In this procedure, leaf disks, leaves, or whole plants are submerged in a beaker containing the solution, and the beaker is placed in a vacuum chamber. The vacuum is then applied, forcing air out of the stomata. When the vacuum is released, the pressure difference forces solution through the stomata and into the mesophyll. Once inside the leaf the *Agrobacterium* remains in the intercellular space and transfers the elafin-Fc coding sequence in high copy numbers into the plant cells. The gene is then transiently expressed (*e.g.*, no selection for stable integration is performed).

[0072] In a specific embodiment of the invention, the transformed agrobacteria are cultured and used to infect 4 to 5-week-old *Nicotiana benthamiana* plants. The leaves of a 4-5 week old *Nicotiana benthamiana* plant are then vacuum infiltrated as described above. Infection takes place either in the presence or absence of a viral RNA gene silencing suppressor. After four days, plant tissue is harvested, homogenized, extracted and tested for amount of protein expression.

H. Protein expression and purification

[0073] Plant biomass can be harvested and ground and elafin-Fc fusion proteins can be recovered using any suitable extraction buffer. An ultrafiltration can be performed to separate the fusion protein and prepare the extract for protein capturing. Affinity chromatography using Protein A resin is typically performed to purify the elafin-Fc fusion protein.

I. Characterization of the plant-made elafin-Fc fusion protein (“PMEF”)

Proteolytic cleavage and glycosylation

[0074] In some embodiments, the purified protein is analyzed for protein purity and coverage using standard techniques such as LC-MS/MS analysis and SDS-PAGE electrophoresis. In addition, site-specific N-glycopeptide analysis using LC-MS/MS can be performed to monitor the glycosylation pattern of the Fc region of the PMEF [28], for example, to confirm that the fusion protein is both galactosylated and sialyated at the N-glycan site (*e.g.*, position 72 of SEQ ID NO:4) in the Fc region to maximize the anti-inflammatory efficacy of the fusion protein [25].

Oxidation

[0075] Oxidation of amino acid residues, for example, methionine residues at positions 25 and 51 in the elafin region (SEQ ID NO:1) of the PMEF, can be analyzed in assay mixtures containing physiologically-relevant concentrations of one or more oxidation agents such as H_2O_2 and HOCl [29], xenobiotics such as paraquat, CCl4, and acetaminophen, cigarette smoke, reduced transition metals such as Fe21 or Cu1, γ-irradiation in the presence of O_2, activated neutrophils, ultraviolet (UV) light, ozone, oxidoreductase enzymes, or N-chlorosuccinimide. Methionine residues under these conditions are oxidized to produce methionine sulfoxide, which can be detected by CNBr cleavage/amino acid analysis [60]. Elafin-Fc fusion proteins of the invention containing the mutations selected from the group consisting of M25L and M51V of SEQ ID NO:1 have less oxidation as compared to a control fusion protein that does not have these mutations.

Half-life

[0076] The half-life of the PMEF can be measured using standard techniques, such as pulse-chase analysis and cycloheximide blocking [61]. The PMEF described herein has a longer half-life as compared to native elafin, *e.g.*, at least 2 fold, 3 fold, 5 fold, or at least 10 fold longer. In some embodiments, the PMEF has a half-life in plasma of at least 1 day, at least 3 days, at least 5 days, at least 7 days, at least 10 days, at least 12 days, at least 15 days, at least 18 days, at least 21 days, or at least 25 days.

Activity in inhibiting neutrophilic elastase

[0077] Elafin's inhibitory function on neutrophilic elastase ("NE") can be readily assessed by monitoring the hydrolyzation of a substrate of NE in the presence of PMEF. The hydrolyzation of substrate will be inhibited or stopped in the presence of a PMEF described herein. Many commercial kits are available for testing the inhibitory activity on human neutrophil elastase, for example, the Neutrophil Elastase Inhibitor Screening Kit (ab118971) from Abcam and K782-100 from Biovision.

[0078] Alternatively, the NE-inhibitory function of the PMEF can be assessed using sputum expectorated from adult CF patients. CF sputum is an exemplary matrix representing conditions within the inflamed airway since it contains abundant levels of neutrophils that harbor multiple serine proteases that are inhibited by elafin (elastase, proteinase 3, and cathepsin G). CF sputum contains proteins derived from not only neutrophils, but also those secreted by airway epithelial cells, that are the *in vivo* substrates of neutrophil-derived serine proteases. Thus, this system is more relevant to the human inflammatory airway disease conditions as compared to commercial kits described above. In one particular assay format, spontaneously expectorated sputum from adult CF patients are obtained, divided into aliquots and incubated for various periods of time (0-6 hrs) in the absence or presence of the PMEF. At various intervals, small peptides derived from the activity of endogenous neutrophil proteases are isolated by centrifugation of whole CF sputum through 5 kD molecular weight filters. The isolated peptides are then subjected to LC/MS/MS analysis to determine the sequence, the protein from which the peptides were derived, and quantitative assessment of the proteolytic peptides by previously established methods [30]. This approach using CF sputum thus allows for assessing the efficacy of the PMEF to inhibit neutrophil-derived serine proteases in a complex matrix representative of the inflamed human airway.

J. Targeted diseases and evaluating the efficacy of PMEF therapy

[0079] PMEF can be used to treat inflammatory diseases, *e.g.*, an inflammatory pulmonary disease, inflammatory vascular disease, inflammatory systemic disease, and inflammation triggered by reperfusion injury. In particular embodiments, PMEF can be used to treat an inflammatory pulmonary disease that is selected from the group consisting of alpha-1 antitrypsin deficiency (AATD), cystic fibrosis (CF), and chronic obstructive pulmonary disease (COPD).

[0080] AATD is diagnosed by a showing of low serum concentration of alhpha antitrypsin (AAT) and either detection of a functionally deficient AAT protein variant or detection of biallelic pathogenic variants in the gene encoding alpha-1 antitrypsin [62]. AATD patients typically show one or more of the following symptoms: shortness of breath following mild activity, reduced ability to exercise, wheezing, unintentional weight loss, recurring respiratory infections, fatigue, and rapid heartbeat upon standing. The lack of functional AAT resulted from the AATD in these patients often leads to chronic obstructive pulmonary disease (COPD), which include Emphysema, a type of COPD caused by damage to the small air sacs in the lungs. Patients having emphysema often show one or more of the following symptoms: difficulty in breathing, a hacking cough, and a barrel-shaped chest.

[0081] CF can be diagnosed with a number of tests. For example, all states screen newborns using a genetic test to detect the presence of faulty cystic fibrosis transmembrane conductance regulator (CFTR). Mutations in CFTR alter the production, structure, or stability of the chloride channel, which impairs the transport of chloride ions and the movement of water into and out of cells. As a result, cells that line the passageways of the lungs, pancreas, and other organs produce mucus that is abnormally thick and sticky. The abnormal mucus obstructs the airways and glands, leading to the characteristic signs and symptoms of cystic fibrosis.

[0082] A blood test can also be performed on a newborn to diagnose the presence of CF by showing whether the pancreas is working properly. If a genetic test or blood test suggests CF, a sweat test, which measures the amount of salt in sweat is performed; high salt level confirm a diagnosis of CF. Additional tests such as a chest x-ray, which shows whether the lung is inflamed or scarred, or whether they trap air; a sinus x-ray, which may show signs of sinusitis, a complication of CF; lung function tests, which measure how much air the patient can breathe in and out, how fast the patient can breathe air out, and how well the lung deliver oxygen to the blood. CF patients often show one or more of the following symptoms: difficulty breathing and coughing up mucus as a result of frequent lung infections, sinus infections, poor growth, fatty stool, clubbing of the fingers and toes, and infertility in males.

[0083] PMEF therapy is expected to relieve the symptom of the diseases described above by *e.g.,* inhibiting the function of NE, which are present in the respiratory tract secretions of the patients having the diseases described above [3]. Methods for evaluating the efficacy of the PMEF therapy are well known to the medical professionals treating these inflammatory diseases. For example, the clinical benefits for CF or AATD patients receiving PMEF therapy, which correspond to the efficacy of the PMEF, can be measured by one or more of the following criteria: an alleviation of symptoms that are commonly associated with the disease, including, but not limited to the symptoms described above; a decrease in frequencies in lung infections; a reduction in the amount of mucus present in the lungs; a decrease in intestinal blockage, and pulmonary function test results which indicate an improvement in lung function. Non-limiting examples of lung function tests include spirometry, lung volumes test, and diffusion capacity test.

K. Pharmaceutical compositions comprising PMEF

[0084] The PMEFs of the present invention are useful in the manufacture of a pharmaceutical composition or a medicament for treating inflammatory diseases as described above. Pharmaceutical compositions or medicaments for use in the present invention can be formulated by standard techniques using one or more physiologically acceptable carriers or excipients. Suitable pharmaceutical carriers are described herein and in, *e.g.,* “Remington's

Pharmaceutical Sciences" by E.W. Martin. PMEFs of the present invention and their physiologically acceptable salts and solvates can be formulated for administration by any suitable route, including, but not limited to, orally, topically, nasally, rectally, parenterally (*e.g.*, intravenously, subcutaneously, intramuscularly, *etc.*), and combinations thereof. In some embodiments, the therapeutic agent is dissolved in a liquid, for example, water.

[0085] For oral administration, a pharmaceutical composition or a medicament disclosed herein can take the form of, *e.g.*, a tablet or a capsule prepared by conventional means with a pharmaceutically acceptable excipient. Preferred are tablets and gelatin capsules comprising the active ingredient(s), together with (a) diluents or fillers, *e.g.*, lactose, dextrose, sucrose, mannitol, sorbitol, cellulose (*e.g.*, ethyl cellulose, microcrystalline cellulose), glycine, pectin, polyacrylates and/or calcium hydrogen phosphate, calcium sulfate, (b) lubricants, *e.g.*, silica, anhydrous colloidal silica, talcum, stearic acid, its magnesium or calcium salt (*e.g.*, magnesium stearate or calcium stearate), metallic stearates, colloidal silicon dioxide, hydrogenated vegetable oil, corn starch, sodium benzoate, sodium acetate and/or polyethyleneglycol; for tablets also (c) binders, *e.g.*, magnesium aluminum silicate, starch paste, gelatin, tragacanth, methylcellulose, sodium carboxymethylcellulose, polyvinylpyrrolidone and/or hydroxypropyl methylcellulose; if desired (d) disintegrants, *e.g.*, starches (*e.g.*, potato starch or sodium starch), glycolate, agar, alginic acid or its sodium salt, or effervescent mixtures; (e) wetting agents, *e.g.*, sodium lauryl sulfate, and/or (f) absorbents, colorants, flavors and sweeteners. In some embodiments, the tablet contains a mixture of hydroxypropyl methylcellulose, polyethyleneglycol 6000 and titatium dioxide. Tablets may be either film coated or enteric coated according to methods known in the art.

[0086] Liquid preparations for oral administration can take the form of, for example, solutions, syrups, or suspensions, or they can be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations can be prepared by conventional means with pharmaceutically acceptable additives, for example, suspending agents, for example, sorbitol syrup, cellulose derivatives, or hydrogenated edible fats; emulsifying agents, for example, lecithin or acacia; non-aqueous vehicles, for example, almond oil, oily esters, ethyl alcohol, or fractionated vegetable oils; and preservatives, for example, methyl or propyl-p-hydroxybenzoates or sorbic acid. The preparations can also contain buffer salts, flavoring, coloring, and/or sweetening agents as appropriate. If desired, preparations for oral administration can be suitably formulated to give controlled release of the active compound.

[0087] For topical administration, the compositions of the present invention can be in the form of emulsions, lotions, gels, creams, jellies, solutions, suspensions, ointments, and transdermal patches. For delivery by inhalation, the composition can be delivered as a dry powder or in liquid form via a nebulizer. For parenteral administration, the compositions can be in the form of sterile injectable solutions and sterile packaged powders. Preferably, injectable solutions are formulated at a pH of about 4.5 to about 7.5.

[0088] The compositions of the present invention can also be provided in a lyophilized form. Such compositions may include a buffer, *e.g.,* bicarbonate, for reconstitution prior to administration, or the buffer may be included in the lyophilized composition for reconstitution with, *e.g.,* water. The lyophilized composition may further comprise a suitable vasoconstrictor, *e.g.,* epinephrine. The lyophilized composition can be provided in a syringe, optionally packaged in combination with the buffer for reconstitution, such that the reconstituted composition can be immediately administered to a patient.

[0089] The compounds can be encapsulated in a controlled drug-delivery system such as a pressure controlled delivery capsule [63], a colon targeted delivery system, a osmotic controlled drug delivery system, and the like. The pressure controlled delivery capsule can contain an ethylcellulose membrane. The colon target delivery system can contain a tablet core containing lactulose which is over coated with an acid soluble material, *e.g.*, Eudragit E®, and then overcoated with an enteric material, *e.g.*, Eudragit L®. The osmotic controlled drug delivery system can be a single or more osmotic unit encapsulated with a hard gelatin capsule (*e.g.*, capsule osmotic pump; commercially available from, *e.g.*, Alzet, Cupertino, CA). Typically, the osmotic unit contains an osmotic push layer and a drug layer, both surrounded by a semipermeable membrane.

L. Dosage

[0090] Pharmaceutical compositions or medicaments can be administered to a subject at a therapeutically effective dose to treat the inflammatory diseases as described herein. In some embodiments, the pharmaceutical composition or medicament is administered to a subject in an amount sufficient to elicit an effective therapeutic response in the subject.

[0091] Generally, administered dosages will be effective to deliver picomolar to micromolar concentrations of the active ingredients of the compositions to the appropriate site or sites. However, one of ordinary skill in the art understands that the dose administered will vary depending on a number of factors, including, but not limited to, the subject's body weight, age, individual condition, surface area or volume of the area to be treated, and/or on the form of administration. The size of the dose also will be determined by the existence, nature, and extent of any adverse effects that accompany the administration of a particular compound in a particular subject. Preferably, the smallest dose and concentration required to produce the desired result should be used. Dosage should be appropriately adjusted for children, the elderly, debilitated patients, and patients with cardiac and/or liver disease. Further guidance can be obtained from studies known in the art using experimental animal models for evaluating dosage.

[0092] A unit dosage for oral administration to an individual (*e.g.*, human) of about 50 to 70 kg may contain between about 20 and 300 mg of the active ingredient. Typically, a dosage of the active compounds is a dosage that is sufficient to achieve the desired effect. Optimal dosing schedules can be calculated from measurements of agent accumulation in the body of a subject. In general, dosage may be given once or more daily, weekly, or monthly. Persons of ordinary skill in the art can easily determine optimum dosages, dosing methodologies, and repetition rates.

[0093] In some embodiments, the compositions of the invention are administered one or more times a day, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more times a day.

[0094] In some embodiments, the compositions of the invention are administered for about 1 to about 31 days, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, or 31 days. In some embodiments, the compositions of the invention are administered for at least 1 day. In other embodiments, the compositions of the invention are administered for one or more weeks, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or more weeks. In yet other embodiments, the compositions are administered for one or more months, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more months.

[0095] To achieve the desired therapeutic effect, the compositions of the invention may be administered for multiple days at the therapeutically effective daily dose. Thus, therapeutically effective administration of the compositions of the invention to treat a pertinent condition or disease described herein in a subject requires periodic (*e.g.*, daily or twice daily) administration that continues for a period ranging from three days to two weeks or longer. While consecutive daily doses are a preferred route to achieve a therapeutically effective dose, a therapeutically beneficial effect can be achieved even if the agents are not administered daily, so long as the administration is repeated frequently enough to maintain a therapeutically effective concentration of the agents in the subject. For example, one can administer the agents every day, every other day, or, if higher dose ranges are employed and tolerated by the subject, twice a week.

[0096] A dose can be formulated in animal models to achieve a concentration range that includes the IC_{50} (the concentration of the agent that achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in stool or an enteric tissue sample can be measured, for example, by high performance liquid chromatography (HPLC). In general, the dose equivalent of the active ingredient of the composition of the invention is from about 1 ng/kg to about 100 mg/kg for a typical subject.

[0097] The dosage of a composition of the present invention can be monitored and adjusted throughout treatment, depending on severity of symptoms, frequency of recurrence, and/or the physiological response to the therapeutic regimen. Those of skill in the art commonly engage in such adjustments in therapeutic regimens.

IV. EXAMPLES

[0098] The present invention will be described in greater detail by way of specific examples. The following examples are offered for illustrative purposes, and are not intended to limit the invention in any manner. Those of skill in the art will readily recognize a variety of noncritical parameters which can be changed or modified to yield essentially the same results.

EXAMPLE 1. METHODS

1. Cloning

[0099] A gene coding for the human elafin followed by a hinge region (human IgG2) and two serines and then the coding sequence of the Fc domain from human IgG1 was codon-optimized for *Nicotiana benthamiana* expression. A nucleotide sequence encoding the Ramy3D signal peptide was fused to the N-terminal of the elafin coding region to enable secretion to the plant apoplast. Also, nucleotides encoding the omega leader sequence were included between the start of the coding region and the CaMV 35S promoter to improve elafin-Fc protein expression. The binary expression vector (pDP16.0707) that was created as a consequence was transformed into *Agrobacterium tumefaciens* EHA105 via electroporation. The expression vector encodes the elafin-Fc fusion protein corresponding to SEQ ID NO:13.

2. Preparation of *Nicotiana benthamiana* Plants

[0100] Wild-type *Nicotiana benthamiana* seedlings were grown from seed in soil-filled 4-inch pots in the greenhouse. Two weeks after germination, seedlings were transplanted into 4-inch pots and the soil was supplemented with Osmocote fertilizer (Scotts Miracle-Gro Company, Marysville, OH, USA). All of the *Nicotiana benthamiana* plants were grown in the greenhouse with a 16-h photoperiod with the optimal temperature of 30°C (daytime high) and 18°C (night time low) for four to six weeks.

3. Bacterial Preparation

[0101] Recombinant *Agrobacterium tumefaciens* cells were grown overnight in 10 mL of Luria-Bertani (LB) broth containing appropriate selection antibiotics. For each culture, 2.5 mL was then transferred to 1000 mL flasks containing 250 mL of LB media and grown overnight at 28 °C with 250 rpm shaking. Bacterial cells were harvested by centrifugation at 2600xg for 30 min and resuspended in sterile 10 mM MES buffer (pH 5.6) (Fisher Scientific, Santa Clara, CA, USA) containing 10 mM MgCl2 and 150 μM acetosyringone (Sigma-Aldrich, St. Louis, MO, USA). The cell density of the resuspended agrobacterial strains was adjusted to achieve an OD600 of 0.5 for elafin-Fc and p19 strains. These agrobacterial strains were mixed in a 1:1 volume ratio and were incubated in the dark for up to 3 h before infiltration.

4. Agroinfiltration and Plant Incubation

[0102] Five-week old potted greenhouse *Nicotiana benthamiana* plants were inverted and immersed in 1000 mL of the agrobacterial solution having 0.02% of Silwet-L-77 (Lehle Seeds, Round Rock, TX, USA) and placed in a Nalgene container for vacuum infiltration (-25 in Hg) for 2 min before releasing the vacuum. The infiltrated plants were incubated in a controlled environmental growth chamber at 90% humidity and 21°C for five days, and then the leaves were cut at the petioles and harvested. The agroinfiltrated leaves were stored at -80°C for further analysis.

5. Harvested Leaves Agroinfiltration and Incubation

[0103] For the harvested leaves infiltration approach, leaves were cut at the petioles and harvested leaves were submerged in a 200 mL of agrobacterial solution having 0.02% of Silwet-L-77 (Lehle Seeds, Round Rock, TX, USA) and placed in a Nalgene container for vacuum infiltration (-25 in Hg) for 2 min before releasing the vacuum. For the incubation, humidity boxes were prepared using Perlite (E.B. Storage) which was soaked in water before using. The storage box was layered with socked Perlite and then metal mesh was placed on the box as a platform for the leaves. Infiltrated leaves were air dried to remove the surface water before incubation in the humidity boxes. Incubation of humidity boxes was performed under dark condition in a controlled environmental growth chamber at 90% humidity and 21°C for five days, and then the leaves were cut at the petioles and harvested. The agroinfiltrated leaves were stored at -80°C for further analysis.

6. Extraction

[0104] To determine the production level of elafin-Fc protein at five days post-infiltration time point, the biomass was ground in liquid nitrogen at 1:4 ratio (1 g biomass in 4 mL buffer) using phosphate buffered saline (PBS) buffer containing 1 mM EDTA and 2 mM sodium metabisulfite, and incubated for 30 min prior to centrifugation at 2600×g for 30 min. The centrifuged samples were filtered through a 0.22 µm filter. Microfiltered plant extract was then used for quantification or purification.

7. Purification

[0105] Microfiltered plant extract was purified by Protein-A affinity chromatography (MabSelect SuReTM, GE Healthcare, Marlborough, MA, USA). 1.5 mL of Protein-A affinity resin was equilibrated with 10 column volumes of PBS buffer followed by sample load at 1 mL/min flow rate. The resin was then washed with 15 to 20 column volumes of PBS buffer. Elution took place by passing 10 column volumes of 100 mM glycine buffer (pH 2.5) to recover the bound elafin-Fc fusion protein. Finally, elution fractions were neutralized with 0.5 M Tris buffer.

8. ELISA Analysis

[0106] The production level of elafin-Fc fusion protein in *Nicotiana benthamiana* leaves was quantified using an ELISA method. Microplate wells (Costar 3590, Union City, CA, USA) were coated with Protein-A of *Staphylococcus aureus* (Southern Biotech, Birmingham, AL, USA) diluted to 50 µg/mL in PBS Buffer (pH 7.4) and incubated for 1 h at 37 °C. Blocking was achieved with 5% non-fat dry milk prepared in PBS buffer using a 15-min incubation. After incubation, plates were washed three times with phosphate buffered saline tween-20 (PBST), samples and controls were diluted in PBS buffer, and 50 µL of each sample was applied directly to the coated wells. A standard curve was generated with 2.3, 6.9, 20.6, 61.7, 185.2, 555.6, 1666.7, and 5000 ng/mL using pure Fc fusion protein (CMG2-Fc protein) (supplied by Planet Biotechnology, Inc., Hayward, CA, USA) diluted in PBS buffer. Microplates were incubated with 50 µL of diluted samples and standards at 37 °C for 1 h. Then plates were washed three times with PBST buffer and Goat anti-human IgG secondary antibody conjugated with horseradish peroxidase (Southern Biotech) diluted 1:2000 in PBS buffer, which was added. The microplate was incubated for 1 h at 37 °C. Detection was performed with 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Promega, Madison, WI, USA) and the reaction was stopped with 1 N HCl. Finally, the absorbance was measured at 450 nm with a SpectraMax 340C spectrophotometer (Molecular Devices). Each assay was performed in triplicate, and elafin-Fc fusion protein concentrations were interpolated from the linear portion of the standard curve.

9. Dot Blot Analysis

[0107] Dot blot analysis was performed by adding different volume of plant extract to a 0.45 µm nitrocellulose membrane (Bio-Rad). Blots were then air dried for 30 min and blocked with 5% non-fat dry milk (NFDM) prepared in PBS buffer for 1hour at room temperature. The blot was washed 3 times with PBST buffer and incubated with 1:2500 dilution of goat anti-human IgG antibody conjugated with alkaline phosphatase (Southern Biotech) for one hour at room temperature. The blot was washed three times with PBST buffer and developed using AP conjugate substrate kit (Bio-Rad).

10. Immunoblot Analysis

[0108] Protein samples were diluted with 4x Laemmli buffer (Bio-Rad, Hercules, CA, USA) and heated for 5 min at 95°C with 5% β-mercaptoethanol (Bio-Rad) for the reducing gel. Electrophoresis was performed for 35 min at 200 V using 4%-20% gradient gel (Bio-Rad). After electrophoresis was completed, the gels were washed three times with DDH2O. Immunoblot analysis was performed by transferring the gel to a 0.45 µm nitrocellulose membrane (Bio-Rad) at 100 V for 90 min. Blots were then washed with PBST buffer and blocked with 5% non-fat dry milk (NFDM) prepared in PBS buffer for overnight at 4 °C. The blot was incubated with 1:2500 dilution of goat anti-human IgG antibody conjugated with alkaline phosphatase (Southern Biotech) for one hour at room temperature. The blot was washed three times with PBST buffer and developed using AP conjugate substrate kit (Bio-Rad).

11. SDS-PAGE Analysis

[0109] Protein samples were diluted with 4x Laemmli buffer (Bio-Rad, Hercules, CA, USA) and heated for 5 min at 95°C with 5% β-mercaptoethanol (Bio-Rad) for the reducing gel. Electrophoresis was performed for 35 min at 200 V using 4%–20% gradient gel (Bio-Rad). After electrophoresis was completed, the gels were washed three times with DDH2O and stained in Coomassie Brilliant Blue G-250 (Bio-Rad) followed by destaining in DDH2O overnight.

EXAMPLE 2. PRODUCTION, PURIFICATION, AND CHARACTERIZATION OF RECOMBINANT ELAFIN-FC FUSION PROTEIN (VARIANT 5)

[0110] *Nicotiana benthamiana* plants were transformed with the expression vector encoding SEQ ID NO:13, as described in Example 1. The elafin-Fc fusion protein corresponding to SEQ ID NO:13 comprises a codon-optimized human elafin domain was fused to the Fc domain of human IgG1 using two serines and a hinge region as a fusion protein linker. To secrete the elafin-Fc fusion protein to the *Nicotiana benthamiana* apoplast, the rice α-amylase 3D gene signal peptide (Ramy3D) was included at the *N*-terminus of the elafin domain. Also, the Ω leader sequence, which is a modified version of the Ω leader sequence from tobacco mosaic virus and corresponds to SEQ ID NO:16, was included to enhance transient protein production. This protein was expressed under the control of *Cauliflower mosaic virus* (CaMV) 35S promoter. Figure 7C shows the fusion protein expression from harvested leaf. The produced protein was then purified.

[0111] The results show that using the CaMV 35S promoter and co-expression with the *Tomato bushy stunt virus* p19 gene silencing suppressor, an initial expression level of plant-made elafin-Fc fusion protein of over 300 mg/kg fresh leaf biomass was obtained in just 5 days after vacuum agroinfiltration, which is much higher than the production level of the commercially available yeast expression system for elafin production (10 mg/L culture media) [65].

[0112] Downstream bioprocessing of biopharmaceuticals represents some challenge in bioprocess development, and its cost is estimated to range from 65% to 90% of total manufacturing costs depending on the production platform [67]. The Fc domain allows for easy and cost-effective purification by employing Protein A affinity chromatography, a platform technology developed for monoclonal antibody purification. Using this method, under preliminary, non-optimized conditions, we have recovered highly pure plant-made elafin-Fc fusion protein with over 60% recovery, which is higher than that for commercially available yeast-produced, purified elafin (20.3%) [65]. The purity of the plant-made elafin-Fc fusion protein is estimated to be >98% based on SDS-PAGE electrophoresis (FIG. 7). We have optimized the downstream process for plant-made Fc fusion proteins [66].

[0113] To determine whether the purification process meets those criteria including identity (amino acid sequence), post-translational modifications (*e.g.,* site-specific glycoforms analysis), activity (inhibition of neutrophil elastase), purity (>98%), host cell protein/DNA and endotoxin, the purity of the plant-made elafin-Fc fusion protein was assessed using SDS-PAGE and Western blot analysis. The integrity and N-glycosylation of the molecule can be assessed using mass spectrometry [66].

[0114] The fusion protein was confirmed using Dot-blot experiments performed as described in Example 1 and the results are shown in FIG. 4. The protein production was quantified by ELISA as described in Example 1. The results show that the intact plant produced more than 300 mg/kg of the elafin-Fc fusion protein, while the harvested leaf produced about 150 mg/kg of the elafin-Fc fusion protein (FIG. 5A). The elafin-Fc fusion protein was also detected by Western blots using methods described in Example 1 and the results are shown in FIG. 5B; the left panel shows fusion protein from the intact plant, and the right panel shows the fusion protein from the harvested leaf. To further confirm the production level of the elafin-Fc fusion protein, at the time point that is five days post-infiltration, proteins from intact plant and harvested leaf were extracted, microfiltered, and subjected to protein A affinity chromatography as described above. The elafin-Fc fusion protein was recovered by elution with glycine buffer. The results of the fusion protein from the intact plant are shown in FIG. 6A (chromatogram of the total protein) and FIG. 6B (elution peak of the elafin-Fc fusion protein). The results of the fusion protein from the harvested leaf are shown in FIG. 7A (chromatogram of the total protein) and FIG. 7B (elution peak of the elafin-Fc fusion protein).

EXAMPLE 3. PRODUCTION, PURIFICATION, AND CHARACTERIZATION OF RECOMBINANT ELAFIN-FC FUSION PROTEIN (VARIANT 4)

[0115] Protein engineering offers a solution for loss of stability and biological activity of therapeutic proteins. Using this approach, researchers were able to design target proteins that were resistant to oxidation and proteolytic cleavage [21,22]. Similarly, many biologically active proteins and peptides have a very short serum half-life due to fast liver and/or renal clearance, which limits their exposure in the target tissue and, consequently, their pharmacological effects. The Fc domain prolongs the serum half-life of Fc-fusion proteins due to pH-dependent binding to the neonatal Fc receptor (FcRn), which salvages the protein from being degraded in endosomes. In addition, from a processing viewpoint, the Fc region allows for an easy cost-effective purification by Protein-G/A affinity chromatography during manufacture [23,24]. However, the limitation of using this expression platform for glycoprotein production is lack of the sialyation pathway. Although native elafin is not glycosylated, the Fc region is glycosylated. Sialyation in the Fc domain was demonstrated to be beneficial in suppression of inflammation using IgG therapy [25]. Thus, engineering the *N*-glycosylation pathway in plants represents a new production technology platform to address this limitation [26]. This example describes the development of an integrated approach to generate engineered novel elafin-Fc fusion proteins transiently produced via a CaMV 35S expression system in glycoengineered *Nicotiana benthamiana* plants.

[0116] In addition, in order to efficiently produce the elafin gene, a mammalian protein, in plants, the elafin gene was codon-optimized for expression in *N. benthamiana* using the codon usage table for this plant from the KEGG database. A codon-optimized elafin-Fc gene, SEQ ID NO:10, was designed for expression in *Nicotiana benthamiana* (GenScript, Piscataway, NJ 08854, USA). Table 3 shows codon optimization of the elafin gene for expression in *N. benthamiana* or *Pichia pastoris*. In particular, the codon-optimized elafin sequence for expression in *N. benthamiana* is the elafin region of SEQ ID NO:10 and the codon-optimized elafin sequence for expression in *Pichia pastoris* is set forth in SEQ ID NO:11. The codon-optimized elafin-Fc fusion protein gene, *e.g.*, SEQ ID NO:10, can then be synthesized.

Table 3. Codon Optimization

Parameters	**Codon optimized elafin gene (*N. benthamiana* expression)**	**Codon optimized elafin gene (*Pichia* expression system)**
Codon adaptation index (CAI)	0.94	0.82
Frequency of optimal codons (FOP)	75%	57%
GC content adjustment (GC)	42.43	39.19

Production of elafin-Fc fusion model protein in plants

[0117] Our goal is to produce elafin-Fc fusion protein with terminal galactosylation by using an apoplast targeting signal peptide. Tobacco is an efficient expression host since it grows fast, has a high biomass density with large porous leaves making it ideal for agroinfiltration. An elafin-Fc fusion protein (SEQ ID NO:7) is produced using a CaMV 35S expression vector by transient expression [27]. The monomeric form of this fusion protein is 34.5 kD and is retained in the endoplasmic reticulum. Elafin-Fc protein production is measured at 4 days post-infiltration in five-week old *Nicotiana benthamiana* plants. In this approach, a viral RNA gene silencing suppressor (P19) is co-expressed to improve eElafin-Fc fusion protein production.

Purification of plant-made elafin-Fc fusion protein (PMEF)

[0118] Plant biomass from the elafin-Fc expressing *Nicotiana benthamiana* plants is ground using liquid nitrogen. 1X PBS buffer is used as the extraction buffer to recover the elafin-Fc protein. A microfiltration step is performed with a 0.22 µm filter followed by ultrafiltration with a 30kD MWCO filter which is used to prepare the extract for protein capturing. Protein A resin (GE Healthcare) is used for the affinity chromatography step. The purity of the Elafin-Fc fusion protein is expected to be >98% based on SDS-PAGE electrophoresis.

Protein characterization

[0119] The purified elafin-Fc fusion protein is subjected to LC-MS/MS analysis to confirm the protein identity and protein coverage. The results show that 99% coverage of the protein sequence is observed. The site-specific *N*-glycopeptide analysis of purified elafin-Fc model fusion protein is performed according to Nwosu *et al* [28]. The analysis shows that the elafin-Fc fusion protein having complex type *N*-glycan with terminal galactosylation and sialylation at amino acid position 72 of SEQ ID NO:4.

EXAMPLE 4. ASSESSMENT OF THE SAFETY AND EFFICACY OF PLANT-MADE ELAFIN-FC FUSION PROTEIN (PMEF)

[0120] Plant-derived elafin-Fc fusion protein was developed based on three types of mutations: Type 1: mutations engineered on the elafin domain (V5G and V9G) to provide resistance to proteolytic cleavage; Type 2: mutations introduced on the elafin domain (M25L and M51V) to enhance resistance to methionine oxidation; and Type 3: mutations created on the Fc domain (M203L and N209S) increase the serum half-life. Individually, these mutations have been shown not to impact biological activity [36, 37, 68]. To establish the efficacy of plant-derived elafin-Fc fusion protein, *in vitro* methods can be utilized. Elafin-Fc fusion protein can be compared with commercially available yeast produced recombinant elafin. In brief, to determine stoichiometric inhibition of neutrophil elastase by the elafin-Fc fusion protein, equal concentrations of elafin-Fc can be incubated with neutrophil elastase and changes in fluorescence can be monitored using a neutrophil elastase substrate (N-Methoxy-Succinyl-Pro-Ala-Ala-Val-7-amino-4-methylcoumarin) [69]. To determine proteolytic cleavage resistance of elafin-Fc fusion protein, *Pseudomonas* protease and neutrophil elastase can be incubated with elafin-Fc fusion protein and resistance assessed at various incubation time point using Western blot and mass spectrometry analysis [70]. To determine oxidation resistance of elafin-Fc fusion protein, different concentrations of H_2O_2 can be incubated with elafin-Fc fusion protein and resistance assessed using a neutrophil elastase inhibition assay and mass spectrometry [71]. To determine the increased serum half-life of the elafin-Fc fusion protein, neonatal Fc receptor binding efficacy of elafin-Fc fusion protein can be estimated using an ELISA method [72]. The plant-made elafin-Fc fusion protein described herein is oxidant-resistant, has a long circulatory half-life, and is a cost-effective and safe therapy for lung disease, providing a homogeneous and expandable supply of therapeutics for inflammatory lung disease at a lower cost.

[0121] Any potential therapeutic agent requires basic *in vitro* cellular and animal toxicity profiles in the early stages of development. This example further describes performing cell incubation studies of the recombinant elafin-Fc fusion protein variants with human bronchial epithelial (HBE) cells. These cells can be used for our initial toxicology studies. In order to examine safety aspects, the toxicological profiles of elafin-Fc fusion protein can be studied using HBE cells in a dose and time dependent manner. In addition, the biological response of HBE cells can be assessed when exposed to elafin-Fc fusion protein using proteomic approaches. From an efficacy standpoint, inhibition kinetics of elastase can be tested under normal and oxidant exposed elafin-Fc fusion protein when incubated with the HBE cell line.

(i) Assessing the resistance of elafin-Fc fusion protein to inactivation by inflammatory oxidants hydrogen peroxide and hypochlorous acid

[0122] Residual human neutrophil elastase (HNE) or porcine pancreatic elastase (PPE) activity assays can be performed to determine the efficacy of elafin-Fc variants in inhibiting elastase. Yeast recombinant elafin and commercial available AAT can be used as controls for this assay. Oxidation resistance can be determined for the plant-made elafin-Fc fusion variants and the control while incubating them in a assay buffer containing physiologically relevant concentrations of H_2O_2 and HOCl [29].

(ii) Assessing the *ex vivo* efficacy of elafin-Fc variants to inhibit neutrophil-derived serine proteases in sputum from CF patients using "peptidomics" mass spectrometry.

[0123] In order to expand the assessment of elafin-Fc fusion protein efficacy to more complicated systems directly relevant to inflammatory airway disease conditions, sputum expectorated from adult CF patients can be used. CF sputum is an exemplary matrix representing conditions within the inflamed airway since it contains abundant levels of neutrophils that harbor multiple serine proteases that are inhibited by elafin (*e.g.*, elastase, proteinase 3, and cathepsin G). CF sputum contains proteins derived from not only neutrophils, but also those secreted by airway epithelial cells, that are the *in vivo* substrates of neutrophil-derived serine proteases. Spontaneously expectorated sputum from adult CF patients can be obtained, divided into aliquots and incubated for various periods of time (0-6 hrs) in the absence or presence of elafin-Fc variants. At various intervals, small peptides derived from the activity of endogenous neutrophil proteases can be isolated by centrifugation of whole CF sputum through 5 kD molecular weight filters. The isolated peptides can then be subjected to LC/MS/MS analysis to determine the sequence, the protein from which the peptides were derived, and quantitative assessment of the proteolytic peptides by previously established methods [30]. This approach using CF sputum allows for assessing the efficacy of elafin-Fc fusion protein to inhibit neutrophil-derived serine proteases in a complex matrix representative of the inflamed human airway, and establish a procedure for defining the inflamed airway 'peptidome'. In addition, the approach has added value in that it can elucidate novel 'biomarkers' of inflammatory airway diseases.

EXAMPLE 5. DEVELOPMENT OF A NOVEL THERAPY FOR LUNG DISEASE USING A MOUSE MODEL

[0124] Chronic obstructive pulmonary disease (COPD), manifested as emphysema and chronic airway obstruction, can be exacerbated by bacterial and viral infections. Although the frequency of exacerbations increases as the disease progresses, the mechanisms underlying this phenomenon are largely unknown, and there is a need for a simple *in vivo* exacerbation model. Mice exposed to tobacco smoke may represent a novel model of tobacco smoke-induced lung inflammation and disease. However, this approach is not only time consuming but also costly. Thus, in this study, a simple *in vivo* exacerbation model of

COPD can be established by a single administration of elastase followed by lipopolysaccharide (LPS). This simple mouse model mimics human cases and can be useful for elucidating its mechanism and developing therapeutic strategies. In this example, plant-made elafin-Fc fusion proteins can be tested on an elastase and LPS exposed mice model. An exemplary experimental protocol is as follows: Mice aged 10 to 14 weeks are anesthetized by intraperitoneal injection of 1.25% (wt/vol) tribromoethanol, followed by spraying of 4.2 U of porcine pancreatic elastase (PPE) dissolved in 100 ml of sterile PBS or 100 ml of PBS alone (control animals) into the trachea using a MicroSprayer drug delivery device. After 21 days, mice are given intratracheal administration of LPS (1 mg/kg) dissolved in 100 ml of sterile PBS into the trachea of the control and PPE-exposed mice [31-34]. Efficacy of plant made elafin-Fc fusion protein variants can be compared with commercially available yeast-derived elafin protein in terms of serum circulatory half-life, oxidation resistance and proteolytic cleavage resistance. In order to find an optimal drug administration route, the mouse model can be treated by either inhalation or intraperitoneal injection (IP) with elafin-Fc fusion protein variants and controls.

EXAMPLE 6. TREATING A HUMAN PATIENT WITH LUNG DISEASE USING PMEF

[0125] A female patient has been diagnosed with CF since birth. She has difficulty breathing and often coughs up mucus. She has a history of frequent lung infections, sinus infections, poor growth, fatty stool and she appears to have clubbing of the fingers and toes.

[0126] Her physician prescribes and she is given an intravenous infusion of PMEF with an optimal dose once a week, *e.g.*, using a dosing regimen based on drug efficacy. After a period of four months, the lung inflammation symptoms are lessened, less mucus is present in her lungs and her pulmonary function has improved. She also reports less frequent lung infections.

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[0127] Although the forgoing invention has been described in some detail by way of illustration and example for clarity and understanding, it will be readily apparent to one of ordinary skill in the art in light of the teachings of this invention that certain variations, changes, modifications and substitutions of equivalents may be made thereto without necessarily departing from the spirit and scope of this invention. As a result, the embodiments described herein are subject to various modifications, changes and the like, with the scope of this invention being determined solely by reference to the claims appended hereto. Those of skill in the art will readily recognize a variety of non-critical parameters that could be changed, altered or modified to yield essentially similar results. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims. In addition, each reference provided herein is incorporated by reference in its entirety to the same extent as if each reference was individually incorporated by reference. Where a conflict exists between the instant application and a reference provided herein, the instant application shall dominate.

VI. EXEMPLARY EMBODIMENTS

[0128] Exemplary embodiments provided in accordance with the presently disclosed subject matter include, but are not limited to, the claims and the following embodiments:

1. A fusion protein comprising a signal peptide, an elafin region, a linker, and a Fc region.

2. The fusion protein of embodiment 1, wherein the elafin region comprises a sequence that is at least 80%, 85%, 90%, 95%, or 98% identical to SEQ ID NO:1.

3. The fusion protein of embodiment 1 or 2, wherein the fusion protein comprises one or more point mutations in the elafin region relative to SEQ ID NO:1.

4 The fusion protein of embodiment 3, wherein the fusion protein is more resistant to proteolytic cleavage and/or oxidation compared to a control fusion protein without the one or more point mutations in the elafin region.

5. The fusion protein of embodiment 3 or 4, wherein fusion protein comprises one or more of the following point mutations in the elafin region: V5G, V9G, M25L, and M51V.

6. The fusion protein of embodiment 5, wherein the fusion protein comprises the V5G, V9G, M25L, and M51V point mutations in the elafin region.

7. The fusion protein of any one of the embodiments 1 to 6, wherein the signal peptide comprises a sequence that is at least 80%, 85%, 90%, 95%, or 98% identical to SEQ ID NO:2.

8. The fusion protein of any one of the embodiments 1 to 7, wherein the linker comprises a sequence that is at least 80%, 85%, 90%, 95%, or 98% identical to SEQ ID NO:3 or SEQ ID NO:15.

9. The fusion protein of any one of the embodiments 1 to 8, wherein the Fc region comprises a sequence that is at least 80%, 85%, 90%, 95%, or 98% identical to SEQ ID NO:4.

10. The fusion protein of embodiment 9, wherein the fusion protein comprises one or more point mutations in the Fc region relative to SEQ ID NO:4.

11. The fusion protein of embodiment 10, wherein the fusion protein has a longer half-life compared to a control fusion protein without the one or more point mutations in the Fc region.

12. The fusion protein of embodiment 10 or 11, wherein the fusion protein comprises one or more of the following point mutations in the Fc region: M203L and N209S.

13. The fusion protein of embodiment 12, wherein the fusion protein comprises the M203L and N209S point mutations in the Fc region.

14. The fusion protein of embodiment 1, wherein the fusion protein comprises a sequence that is at least 80%, 85%, 90%, 95%, or 98% identical to any one of SEQ ID NOS:5-7 and 13.

15. The fusion protein of any one of embodiments 1 to 14, wherein the Fc region of the fusion protein is galactosylated and sialylated.

16. A polynucleotide encoding the fusion protein of any one of embodiments 1 to 15.

17. The polynucleotide of embodiment 16, wherein the sequence of the polynucleotide has been codon optimized for expressing the fusion protein in a plant.

18. The polynucleotide of embodiment 17, wherein the plant is a tobacco plant.

19. The polynucleotide of embodiment 18, wherein the tobacco plant is *Nicotiana benthamiana*.

20. The polynucleotide of any one of embodiments 16 to 19, wherein the polynucleotide is at least 80%, 85%, 90%, 95%, or 98% identical to any one of SEQ ID NOS:8-10 and 14.

21. An expression vector comprising the polynucleotide of any one of embodiments 16 to 20.

22. The expression vector of embodiment 21, wherein the expression vector comprises a CaMV 35S promoter.

23. The expression vector of embodiment 21, wherein the expression vector comprises an Omega leader sequence of SEQ ID NO:16.

24. A recombinant cell comprising the polynucleotide of any one of embodiments 16 to 20.

25. The recombinant cell of embodiment 24, wherein the recombinant cell is a plant cell.

26. The recombinant cell of embodiment 25, wherein the plant cell is derived from a tobacco plant.

27. The recombinant cell of embodiment 26, wherein the tobacco plant is *Nicotiana benthamiana*.

28. The recombinant cell of any one of embodiments 24 to 27, wherein the recombinant cell lacks fucosylation and xylosylation pathways and has galactosylation pathways.

29. A plant comprising the recombinant cell of any one of embodiments 24 to 28.

30. The plant of embodiment 29, wherein the plant is a tobacco plant.

31. The plant of embodiment 30, wherein the tobacco plant is *Nicotiana benthamiana.*

32. The plant of any one of embodiments 29 to 31, wherein the plant lacks fucosylation and xylosylation pathways and has galactosylation pathways.

33. A method for producing a fusion protein comprising:

(a) providing a polynucleotide of any one of embodiments 16 to 20,

(b) introducing the polynucleotide into a plant tissue, thereby expressing the fusion protein in the plant tissue, and

(c) recovering the fusion protein from the plant tissue.

34. The method of embodiment 33, wherein the plant tissue expresses a viral RNA gene silencing suppressor.

35. The method of embodiment 33, wherein the polynucleotide is introduced into the plant tissue by *Agrobacterium.*

36. The method of embodiment 35, wherein the polynucleotide is introduced into the plant tissue by vacuum infiltration.

37. The method of embodiment 33, wherein the polynucleotide is introduced into the plant tissue by combining *Agrobacterium* with the plant tissue using transient agroinfiltration to form a mixture and to allow infection of at least one plant cell of the plant tissue, and wherein the fusion protein is recovered from the plant tissue after a period of time sufficient for the plant tissue to transiently express the fusion protein.

38. The method of embodiment 37, wherein the period of time sufficient for the plant tissue to transiently express the fusion protein is at least 3 days, at least 4 days, at least 5 days, or at least 6 days.

39. The method of any one of embodiments 33 to 38, wherein the plant tissue is from a plant that lacks fucosylation and xylosylation pathways and has galactosylation pathways.

40. The method of any one of embodiments 33 to 39, wherein the plant tissue is derived from a tobacco plant.

41. The method of embodiment 40, wherein the tobacco plant is *Nicotiana benthamiana.*

42. A composition comprising the fusion protein of any one of embodiments 1 to 15.

43. The composition of embodiment 42, further comprising a pharmaceutically acceptable carrier or excipient.

44. A method for treating an inflammatory disease in a subject, the method comprising administering to the subject an effective amount of the composition of embodiment 42 or 43.

45. The method of embodiment 44, wherein the inflammatory disease is selected from the group consisting of an inflammatory pulmonary disease, inflammatory vascular disease, inflammatory systemic disease, and inflammation triggered by reperfusion injury.

46. The method of embodiment 45, wherein the inflammatory pulmonary disease is selected from the group consisting of alpha-1 antitrypsin deficiency (AATD), cystic fibrosis (CF), and chronic obstructive pulmonary disease (COPD).

INFORMAL SEQUENCE LISTING

SEQ ID NO:1 (Wild type elafin)
AQEPVKGPVSTKPGSCPIILIRCAMLNPPNRCLKDTDCPGIKKCCEGSCGMACFVPQ

SEQ ID NO:2 (Wild type signal peptide)
MKNTSSLCLLLLVVLCSLTCNSGQA

SEQ ID NO:3 (Linker from human IgG1 hinge region)
EPKSCDKTHT

SEQ ID NO:4 (Wild type Fc)
CPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY
RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS
DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

SEQ ID NO:5 (Variant 2 protein sequence; Native form-without any mutation)
MKNTSSLCLLLLVVLCSLTCNSGQAAQEPVKGPVSTKPGSCPIILIRCAMLNPPNRCLKDTDCPGIKKCCEGSCG
MACFVPQEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVE
VHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALH
NHYTQKSLSLSPGK

SEQ ID NO:6 (Variant 3 protein sequence, wherein the elafin region comprises mutations V5G, V9G, M25L, and M51V relative to SEQ ID NO:1)
MKNTSSLCLLLLVVLCSLTCNSGQAAQEPGKGPGSTKPGSCPIILIRCALLNPPNRCLKDTDCPGIKKCCEGSCG
VACFVPQEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVE
VHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALH
NHYTQKSLSLSPGK

SEQ ID NO:7 (Variant 4 sequence, wherein the elafin region comprises mutations V5G, V9G, M25L, and M51V relative to SEQ ID NO:1; and wherein the Fc region comprises mutations M203L and N209S relative to SEQ ID NO:4)
MKNTSSLCLLLLVVLCSLTCNSGQAAQEPGKGPGSTKPGSCPIILIRCALLNPPNRCLKDTDCPGIKKCCEGSCG
VACFVPQEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVE
VHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVLHEALH
SHYTQKSLSLSPGK

SEQ ID NO:8 (Variant 2 nucleic acid sequence)
ATGAAGAATACTTCTTCACTTTGTCTTTTGCTTTTGGTTGTTCTTTGTTCATTGACATGCAATTCTGGTCAAGCT
GCTCAGGAACCTGTTAAGGGTCCAGTTTCAACTAAACCTGGATCTTGTCCAATTATTCTTATCAGATGCGCTATG
TTGAATCCTCCAAATAGGTGTTTGAAGGATACAGATTGCCCTGGAATTAAGAAATGTTGCGAAGGTTCATGTGGA
ATGGCTTGCTTTGTTCCTCAAGAGCCAAAGTCTTGTGATAAAACTCATACATGTCCTCCATGCCCTGCTCCAGAG
CTTTTGGGTGGACCTTCTGTTTTTCTTTTTCCTCCAAAGCCAAAAGATACTTTGATGATTTCAAGAACTCCTGAA
GTTACATGCGTTGTTGTTGATGTTTCTCATGAAGATCCAGAGGTTAAGTTTAATTGGTATGTTGATGGTGTTGAG
GTTCATAATGCTAAGACAAAACCAAGAGAAGAGCAATATAATTCAACTTACAGGGTTGTTTCTGTTCTTACAGTT
TTGCATCAGGATTGGCTTAATGGTAAAGAGTACAAGTGTAAAGTTTCAAATAAGGCTTTGCCTGCTCCAATCGAA
AAGACTATTTCTAAGGCTAAAGGACAACCTAGAGAGCCACAGGTTTATACACTTCCTCCATCAAGGGATGAATTG
ACTAAGAACCAAGTTTCTCTTACATGCTTGGTTAAAGGTTTTTACCCTTCAGATATTGCTGTTGAATGGGAGTCT
AATGGTCAGCCAGAAAATAACTATAAGACTACACCTCCAGTTCTTGATTCAGATGGTTCTTTCTTTCTTTACTCA
AAGTTGACTGTTGATAAGTCTAGGTGGCAACAGGGAAATGTTTTCTCTTGTTCAGTTATGCATGAGGCTTTGCAT
AACCATTACACACAGAAGTCTCTTTCATTGTCTCCTGGAAAA

SEQ ID NO:9 (Variant 3 nucleic acid sequence)
ATGAAGAATACTTCTTCACTTTGTCTTTTGCTTTTGGTTGTTCTTTGTTCATTGACATGCAATTCTGGACAAGCT
GCTCAGGAACCTGGAAAGGGACCAGGTTCAACTAAACCTGGTTCTTGTCCAATTATTCTTATCAGATGCGCTCTT

TTGAATCCTCCAAATAGGTGTTTGAAGGATACAGATTGCCCTGGAATTAAGAAATGTTGCGAAGGATCATGTGGT
GTTGCTTGCTTTGTTCCTCAAGAGCCAAAGTCTTGTGATAAAACTCATACATGTCCTCCATGCCCTGCTCCAGAG
CTTTTGGGTGGACCTTCTGTTTTTCTTTTTCCTCCAAAGCCAAAAGATACTTTGATGATTTCAAGAACTCCTGAA
GTTACATGCGTTGTTGTTGATGTTTCTCATGAAGATCCAGAGGTTAAGTTTAATTGGTATGTTGATGGTGTTGAG
GTTCATAATGCTAAGACAAAACCAAGAGAAGAGCAATATAATTCAACTTACAGGGTTGTTTCTGTTCTTACAGTT
TTGCATCAGGATTGGCTTAATGGTAAAGAGTACAAGTGTAAAGTTTCAAATAAGGCTTTGCCTGCTCCAATCGAA
AAGACTATTTCTAAGGCTAAAGGTCAACCTAGAGAGCCACAGGTTTATACACTTCCTCCATCAAGGGATGAATTG
ACTAAGAACCAAGTTTCTCTTACATGCTTGGTTAAAGGATTTTACCCTTCAGATATTGCTGTTGAATGGGAGTCT
AATGGTCAGCCAGAAAATAACTATAAGACTACACCTCCAGTTCTTGATTCAGATGGTTCTTTCTTTCTTTACTCA
AAGTTGACTGTTGATAAGTCTAGGTGGCAACAGGGTAATGTTTTCTCTTGTTCAGTTATGCATGAGGCTTTGCAT
AACCATTACACACAGAAGTCTCTTTCATTGTCTCCTGGAAAA

SEQ ID NO:10 (Variant 4 nucleic acid sequence)
ATGAAGAATACTTCTTCACTTTGTCTTTTGCTTTTGGTTGTTCTTTGTTCATTGACATGCAATTCTGGACAAGCT
GCTCAGGAACCTGGAAAGGGACCAGGTTCAACTAAACCTGGTTCTTGTCCAATTATTCTTATCAGATGCGCTCTT
TTGAATCCTCCAAATAGGTGTTTGAAGGATACAGATTGCCCTGGAATTAAGAAATGTTGCGAAGGATCATGTGGT
GTTGCTTGCTTTGTTCCTCAAGAGCCAAAGTCTTGTGATAAAACTCATACATGTCCTCCATGCCCTGCTCCAGAG
CTTTTGGGTGGACCTTCTGTTTTTCTTTTTCCTCCAAAGCCAAAAGATACTTTGATGATTTCAAGAACTCCTGAA
GTTACATGCGTTGTTGTTGATGTTTCTCATGAAGATCCAGAGGTTAAGTTTAATTGGTATGTTGATGGTGTTGAG
GTTCATAATGCTAAGACAAAACCAAGAGAAGAGCAATATAATTCAACTTACAGGGTTGTTTCTGTTCTTACAGTT
TTGCATCAGGATTGGCTTAATGGTAAAGAGTACAAGTGTAAAGTTTCAAATAAGGCTTTGCCTGCTCCAATCGAA
AAGACTATTTCTAAGGCTAAAGGTCAACCTAGAGAGCCACAGGTTTATACACTTCCTCCATCAAGGGATGAATTG
ACTAAGAACCAAGTTTCTCTTACATGCTTGGTTAAAGGATTTTACCCTTCAGATATTGCTGTTGAATGGGAGTCT
AATGGTCAGCCAGAAAATAACTATAAGACTACACCTCCAGTTTTGGATTCAGATGGTTCTTTCTTTCTTTACTCA
AAGTTGACTGTTGATAAGTCTAGGTGGCAACAGGGTAATGTTTTCTCTTGTTCAGTTCTTCATGAGGCTTTGCAT
TCTCATTATACACAGAAGTCTCTTTCATTGTCTCCTGGAAAA

SEQ ID NO:11 (Elafin nucleic acid sequence codon-optimized for expression in *Pichia pastoris*)
GCCCAAGAACCAGTCAAGGGTCCAGTCAGTACAAAGCCAGGTAGTTGCCCAATTATCCTTATTAGATGTGCCATG
TTGAATCCTCCTAACAGATGTTTGAAGGATACTGATTGCCCTGGAATCAAAAAGTGCTGTGAAGGTAGTTGCGGT
ATGGCTTGTTTCGTCCCTCAG

SEQ ID NO:12 (Elafin protein sequence encoded by SEQ ID NO:11)
AQEPVKGPVSTKPGSCPIILIRCAMLNPPNRCLKDTDCPGIKKCCEGSCGMACFVPQ

SEQ ID NO:13 (Variant 5 protein sequence, wherein the elafin region comprises mutations V5G, V9G, M25L, and M51V relative to SEQ ID NO:1; wherein the Fc region comprises mutations M203L and N209S relative to SEQ ID NO:4; and wherein the linker region has a sequence of SEQ ID NO:15)
MKNTSSLCLLLLVVLCSLTCNSGQAAQEPGKGPGSTKPGSCPIILIRCALLNPPNRCLKDTDCPGIKKCCEGSCG
VACFVPQSSERKCCVECPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV
HNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELT
KNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVLHEALHS
HYTQKSLSLSPGK

SEQ ID NO:14 (Variant 5 nucleic acid sequence)
ATGAAAAATACCTCTTCTTTATGTTTGCTCTTGTTGGTGGTGCTCTGCTCTCTCACTTGTAACTCTGGTCAGGCT
GCTCAGGAACCTGGAAAGGGACCTGGTTCTACTAAACCTGGATCATGTCCAATTATTCTTATCAGATGCGCTCTT
TTGAATCCTCCAAATAGGTGTTTGAAGGATACAGATTGCCCTGGTATTAAGAAATGTTGCGAAGGATCTTGTGGT
GTTGCTTGCTTTGTTCCACAATCTTCAGAAAGAAAATGTTGCGTTGAGTGTCCTCCATGCCCTGCTCCAGAGCTT
TTGGGTGGACCTTCTGTTTTTCTTTTTCCTCCAAAGCCAAAAGATACTTTGATGATTTCTAGGACTCCTGAAGTT
ACATGTGTTGTTGTTGATGTTTCACATGAAGATCCAGAGGTTAAGTTTAATTGGTATGTTGATGGAGTTGAGGTT
CATAATGCTAAGACAAAACCAAGAGAAGAGCAATATAATTCTACTTACAGGGTTGTTTCAGTTCTTACAGTTTTG
CATCAGGATTGGCTTAATGGTAAAGAATACAAGTGTAAAGTTTCTAATAAGGCTTTGCCTGCTCCAATCGAAAAG
ACTATTTCAAAGGCTAAAGGTCAACCTAGAGAGCCACAGGTTTATACACTTCCTCCATCTAGGGATGAATTGACT
AAGAACCAAGTTTCACTTACATGCTTGGTTAAAGGATTTTACCCTTCTGATATTGCTGTTGAATGGGAGTCAAAT
GGTCAGCCAGAGAATAACTATAAGACTACACCTCCAGTTCTTGATTCTGATGGTTCTTTCTTTCTTTACTCTAAG
TTGACTGTTGATAAGTCAAGATGGCAACAGGGTAATGTTTTCTCTTGCTCAGTTCTCCACGAGGCACTCCATTCA
CATTACACCCAGAAGTCACTCTCTCTCAGTCCAGGAAAA

SEQ ID NO:15 (Linker from human IgG2 hinge region)
SSERKCCVE

SEQ ID NO:16 (the Ω leader sequence, which is modified based on Tobacco mosaic virus (TMV)omega sequence)
TTCAACAATTACCAACAACAACAAACAACAAACAACATTACAATTACTATTTACAATTACAGTCGAAAGGAAGAA
GCC

WHAT IS CLAIMED IS:

1. A fusion protein comprising a signal peptide, an elafin region, a linker, and a Fc region.

2. The fusion protein of claim 1, wherein the elafin region comprises a sequence that is at least 80%, 85%, 90%, 95%, or 98% identical to SEQ ID NO:1.

3. The fusion protein of claim 1, wherein the fusion protein comprises one or more point mutations in the elafin region relative to SEQ ID NO:1.

4 The fusion protein of claim 3, wherein the fusion protein is more resistant to proteolytic cleavage and/or oxidation compared to a control fusion protein without the one or more point mutations in the elafin region.

5. The fusion protein of claim 3, wherein fusion protein comprises one or more of the following point mutations in the elafin region: V5G, V9G, M25L, and M51V.

6. The fusion protein of claim 5, wherein the fusion protein comprises the V5G, V9G, M25L, and M51V point mutations in the elafin region.

7. The fusion protein of claim 1, wherein the signal peptide comprises a sequence that is at least 80%, 85%, 90%, 95%, or 98% identical to SEQ ID NO:2.

8. The fusion protein of claim 1, wherein the linker comprises a sequence that is at least 80%, 85%, 90%, 95%, or 98% identical to SEQ ID NO:3 or SEQ ID NO:15.

9. The fusion protein of claim 1, wherein the Fc region comprises a sequence that is at least 80%, 85%, 90%, 95%, or 98% identical to SEQ ID NO:4.

10. The fusion protein of claim 9, wherein the fusion protein comprises one or more point mutations in the Fc region relative to SEQ ID NO:4.

11. The fusion protein of claim 10, wherein the fusion protein has a longer half-life compared to a control fusion protein without the one or more point mutations in the Fc region.

12. The fusion protein of claim 10, wherein the fusion protein comprises one or more of the following point mutations in the Fc region: M203L and N209S.

13. The fusion protein of claim 12, wherein the fusion protein comprises the M203L and N209S point mutations in the Fc region.

14. The fusion protein of claim 1, wherein the fusion protein comprises a sequence that is at least 80%, 85%, 90%, 95%, or 98% identical to any one of SEQ ID NOS:5-7 and 13.

15. The fusion protein of claim 1, wherein the Fc region of the fusion protein is galactosylated and sialylated.

16. A polynucleotide encoding the fusion protein of claim 1.

17. The polynucleotide of claim 16, wherein the sequence of the polynucleotide has been codon optimized for expressing the fusion protein in a plant.

18. The polynucleotide of claim 17, wherein the plant is a tobacco plant.

19. The polynucleotide of claim 18, wherein the tobacco plant is *Nicotiana benthamiana*.

20. The polynucleotide of claim 16, wherein the polynucleotide is at least 80%, 85%, 90%, 95%, or 98% identical to any one of SEQ ID NOS:8-10 and 14.

21. An expression vector comprising the polynucleotide of claim 16.

22. The expression vector of claim 21, wherein the expression vector comprises a CaMV 35S promoter.

23. The expression vector of claim 21, wherein the expression vector comprises an Omega leader sequence of SEQ ID NO:16.

24. A recombinant cell comprising the polynucleotide of claim 16.

25. The recombinant cell of claim 24, wherein the recombinant cell is a plant cell.

26. The recombinant cell of claim 25, wherein the plant cell is derived from a tobacco plant.

27. The recombinant cell of claim 26, wherein the tobacco plant is *Nicotiana benthamiana*.

28. The recombinant cell of claim 24, wherein the recombinant cell lacks fucosylation and xylosylation pathways and has galactosylation pathways.

29. A plant comprising the recombinant cell of claim 24.

30. The plant of claim 29, wherein the plant is a tobacco plant.

31. The plant of claim 30, wherein the tobacco plant is *Nicotiana benthamiana*.

32. The plant of claim 29, wherein the plant lacks fucosylation and xylosylation pathways and has galactosylation pathways.

33. A method for producing a fusion protein comprising:

(a) providing a polynucleotide of claim 16,

(b) introducing the polynucleotide into a plant tissue, thereby expressing the fusion protein in the plant tissue, and

(c) recovering the fusion protein from the plant tissue.

34. The method of claim 33, wherein the plant tissue expresses a viral RNA gene silencing suppressor.

35. The method of claim 33, wherein the polynucleotide is introduced into the plant tissue by *Agrobacterium*.

36. The method of claim 35, wherein the polynucleotide is introduced into the plant tissue by vacuum infiltration.

37. The method of claim 33, wherein the polynucleotide is introduced into the plant tissue by combining *Agrobacterium* with the plant tissue using transient agroinfiltration to form a mixture and to allow infection of at least one plant cell of the plant tissue, and wherein the fusion protein is recovered from the plant tissue after a period of time sufficient for the plant tissue to transiently express the fusion protein.

38. The method of claim 37, wherein the period of time sufficient for the plant tissue to transiently express the fusion protein is at least 3 days, at least 4 days, at least 5 days, or at least 6 days.

39. The method of claim 33, wherein the plant tissue is from a plant that lacks fucosylation and xylosylation pathways and has galactosylation pathways.

40. The method of claim 33, wherein the plant tissue is derived from a tobacco plant.

41. The method of claim 40, wherein the tobacco plant is *Nicotiana benthamiana*.

42. A composition comprising the fusion protein of claim 1.

43. The composition of claim 42, further comprising a pharmaceutically acceptable carrier or excipient.

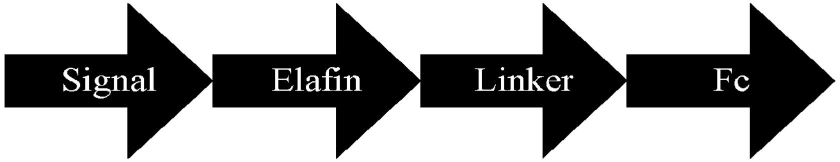
44. A method for treating an inflammatory disease in a subject, the method comprising administering to the subject an effective amount of the composition of claim 42.

45. The method of claim 44, wherein the inflammatory disease is selected from the group consisting of an inflammatory pulmonary disease, inflammatory vascular disease, inflammatory systemic disease, and inflammation triggered by reperfusion injury.

46. The method of claim 45, wherein the inflammatory pulmonary disease is selected from the group consisting of alpha-1 antitrypsin deficiency (AATD), cystic fibrosis (CF), and chronic obstructive pulmonary disease (COPD).

Gene design for elafin-Fc

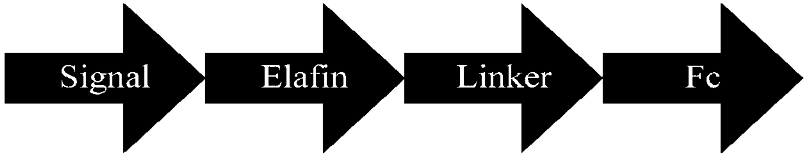
Underline - Signal peptide
Bold solid box - Elafin
Dash - Linker
Dash box - Fc (IgG1)
Asterisk - Mutated
Bold underline - N-Glycan site



MKNTSSLCLLLLVVLCSLTCNSGQA**AQEPGKGPGSTKPGSCPIILIRCALLNP**
PNRCLKDTDCPGIKKCCEGSCGVACFVPQEPKSCDKTHTCPPCPAPELLGGPS
VFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPR
EEQY**N**STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE
PQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVL
DSDGSFFLYSKLTVDKSRWQQGNVFSCSVLHEALHSHYTQKSLSLSPGK

FIG. 1

Gene design for elafin-Fc



Underline	- Signal peptide
Bold solid box	- Elafin
Italic	- Disulfide bonds
Dash	- Linker
Dash box	- Fc (IgG1)
Asterisk	- Mutated
Bold underline	- N-Glycan site

MKNTSSLCLLLLVVLCSLTCNSGQA**AQEPGKGPGSTKPGSCPIILIRCALLN**
PPNRCLKDTDCPGIKKCCEGSCGVACFVPQSSERKCCVECPPCPAPELLGGP
SVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTK
PREEQY**N**STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQ
PREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTT
PPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVLHEALHSHYTQKSLSLSPG
K

FIG. 2

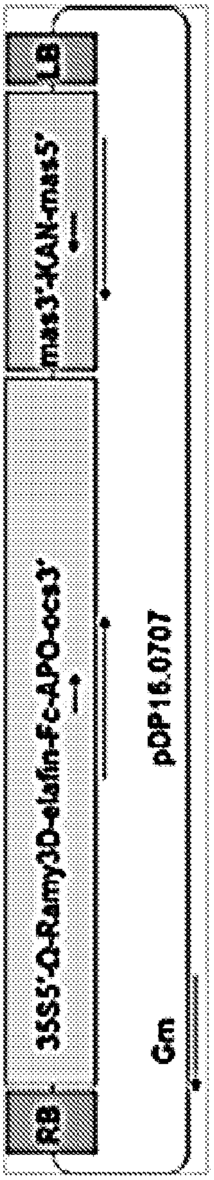

RB
35S5'-O-Ramy3D-elafin-Fc-APO-ocs3'
mas3'-KAN-mas5'
LB
Gm
pDP16.0707

FIG. 3

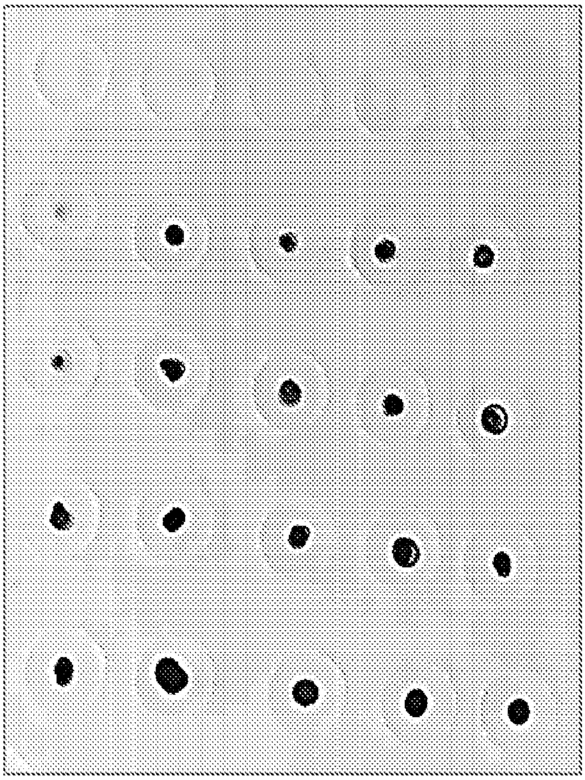


FIG. 4

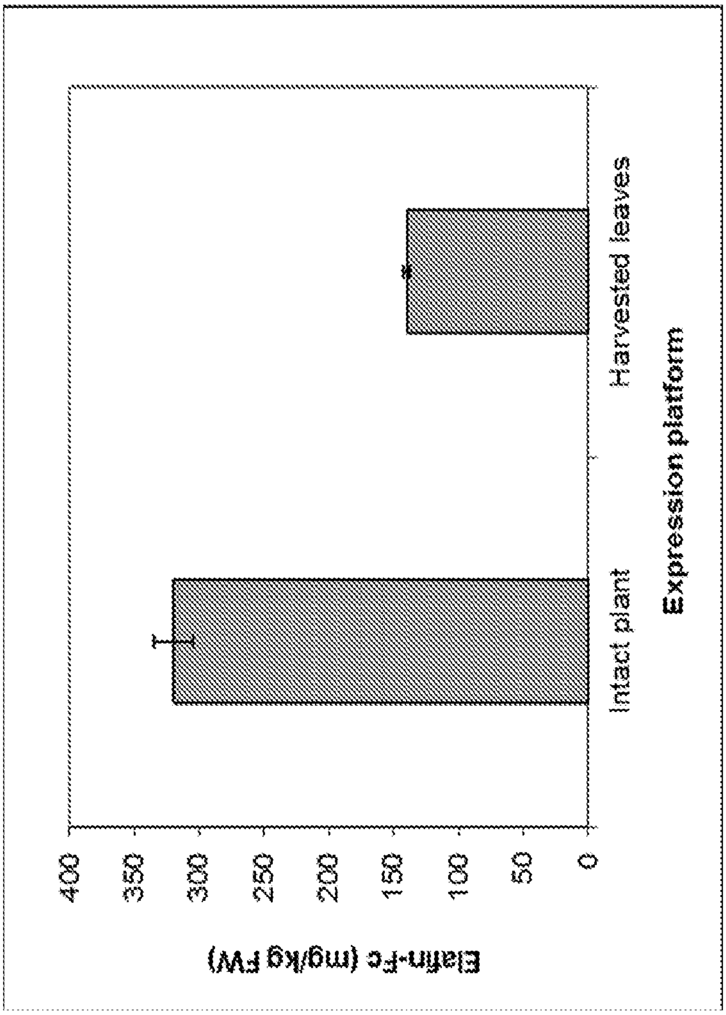

Elafin-Fc (mg/kg FW)
400
350
300
250
200
150
100
50
0
Intact plant
Harvested leaves
*
Expression platform

FIG. 5A

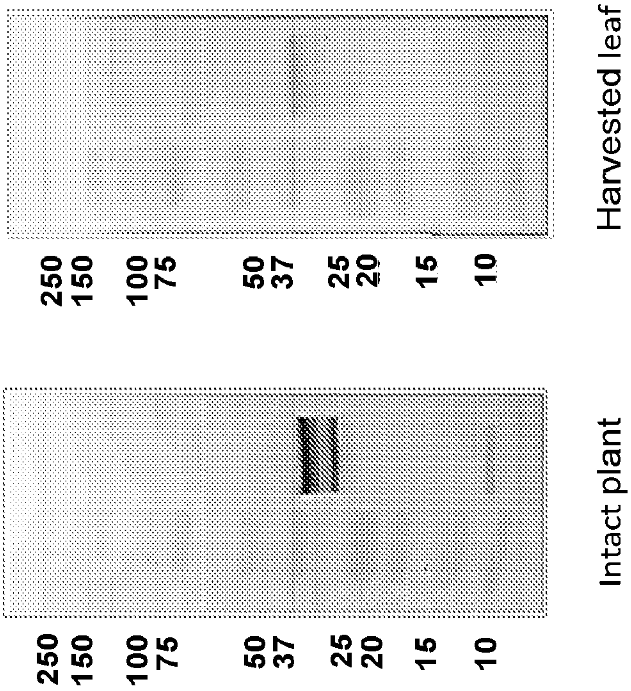

250
150
100
75
50
37
25
20
15
10
Intact plant
250
150
100
75
50
37
25
20
15
10
Harvested leaf

FIG. 5B

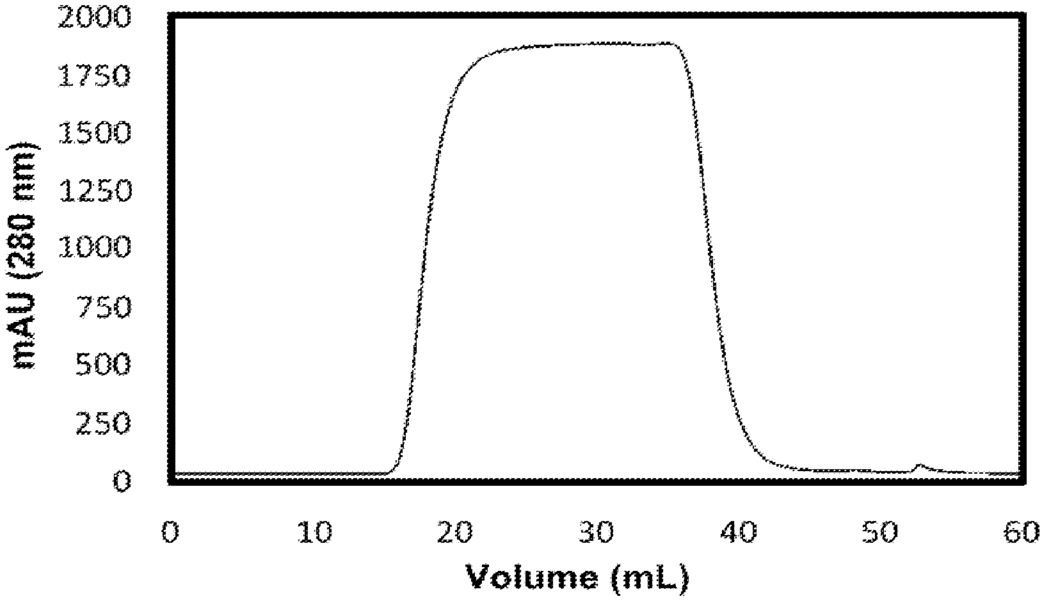

2000
1750
1500
1250
1000
750
500
250
0
mAU (280 nm)
0
10
20
30
40
50
60
Volume (mL)

FIG. 6A

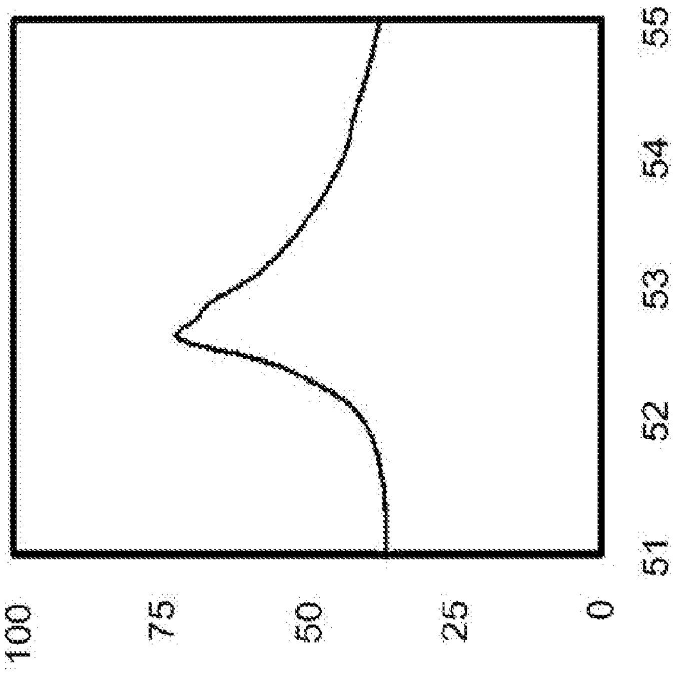
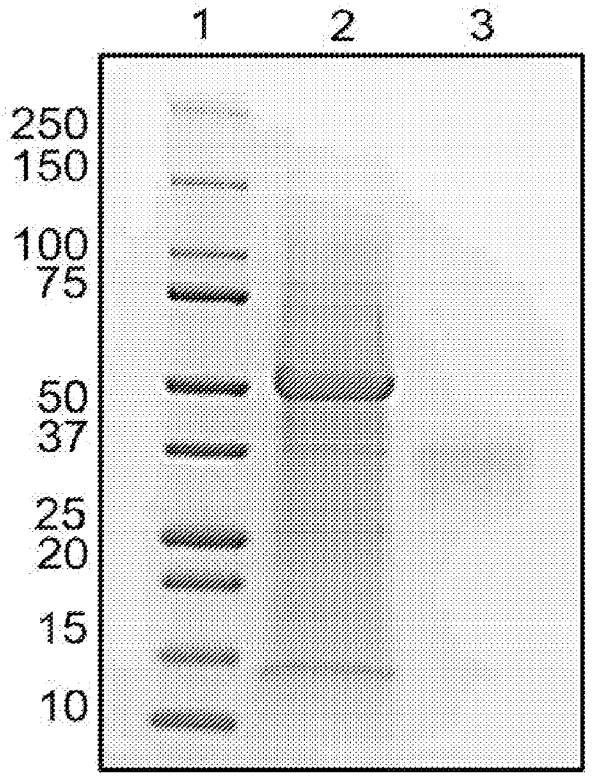


FIG. 6B



SDS-PAGE

Lane 1: Marker
Lane 2: Crude extract 20µl
Lane 3: Elution fraction 30µl

FIG. 6C

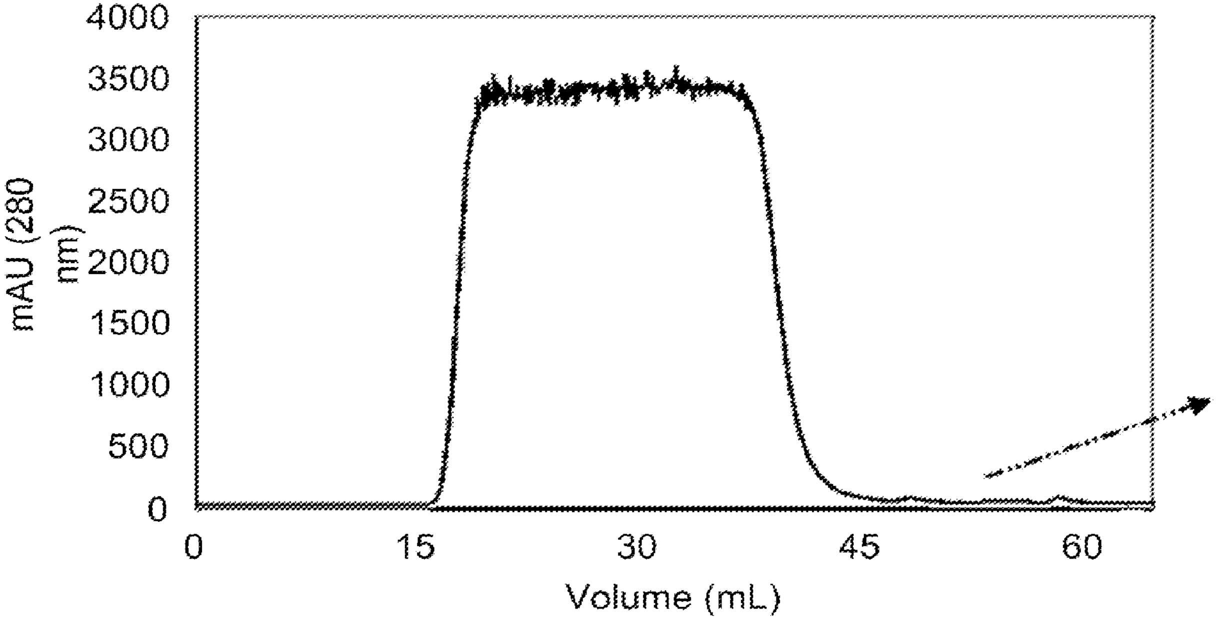

mAU (280 nm)
4000
3500
3000
2500
2000
1500
1000
500
0
0
15
30
45
60
Volume (mL)

FIG. 7A

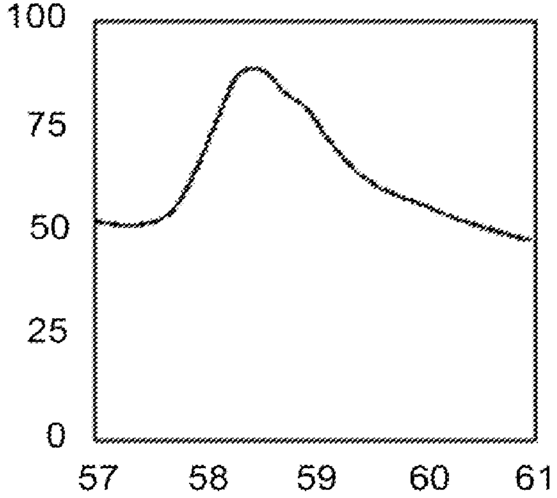
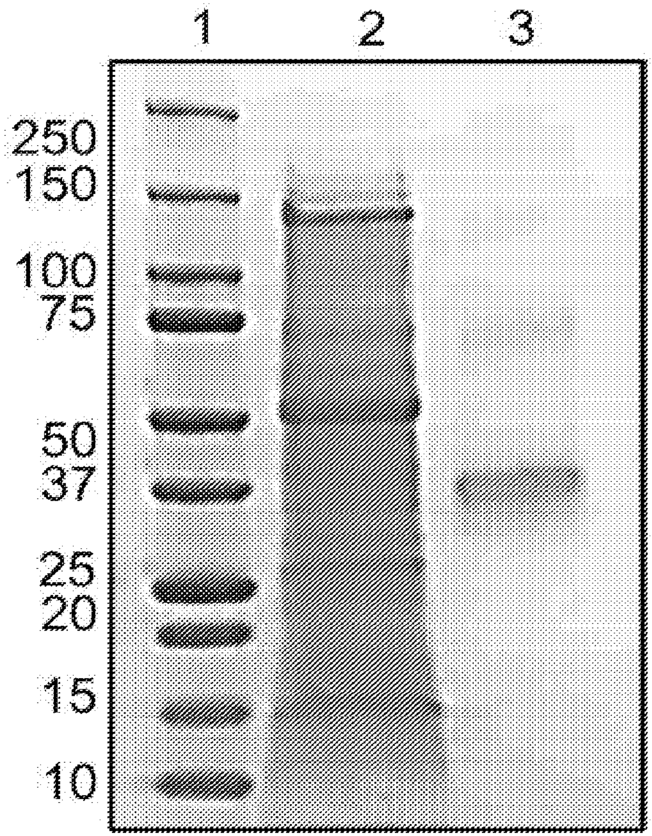

100
75
50
25
0
57
58
59
60
61

FIG. 7B



SDS-PAGE

Lane 1: Marker
Lane 2: Crude extract 20µl
Lane 3: Elution fraction 30µl

FIG. 7C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/30627

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C07K 19/00, 14/81; C12N 15/82, 15/83, 5/04 (2017.01)
CPC - C07K 14/811, 2317/52, 2319/30; C12N 15/82, 15/8257, 5/04

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History Document

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y ---- A	US 2013/0011399 A1 (Timmer et al.) 10 January 2013 (10.01.2013). Especially para [0009], [0082], [0153], claims 3, 14 SEQ ID NO: 21	1, 2, 8, 9, 14, 16, 21, 24, 42, 43 ---- 7, 15, 17-19, 22, 25-32 ---- 20, 23
Y	US 2012/0045818 A1 (Hwang et al.) 23 February 2012 (23.02.2012). Especially para [0009], [0017], [0026-0027], [0066], [0074], sheet 2 fig 2	7, 17-19, 22, 25-27, 29-32
Y	US 2016/0024179 A1 (Pyranose Biotherapeutics, Inc) 28 January 2016 (28.01.2016). Especially para [0015]).	15
Y	Strasser. Engineering of human-type O-glycosylation in Nicotiana benthamiana plants. Bioengineered Jul-Aug 2013 Vol 4 No 4 Pages 191-196. Especially pg 192 col 2 para 1	28, 32
A	US 2016/0017019 A1 (Protalix, Ltd.) 21 January 2016 (21.01.2016). Especially SEQ ID NO: 5	20
A	US 2010/0281567 A1 (Galili et al.) 4 November 2010 (04.11.2010). Especially para [0102], SEQ ID NO: 5	23

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
16 August 2017

Date of mailing of the international search report
26 SEP 2017

Name and mailing address of the ISA/US
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450
Facsimile No. 571-273-8300

Authorized officer:
Lee W. Young
PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/30627

Box No. I **Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

a. ☒ forming part of the international application as filed:

☒ in the form of an Annex C/ST.25 text file.

☒ on paper or in the form of an image file.

b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

GenCore ver 6.4.1 SEQ ID NOs: 1-5, 8, 16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/30627

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

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

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

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

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

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

This International Searching Authority found multiple inventions in this international application, as follows:
-----Go to Extra Sheet for continuation------

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

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

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

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-2, 7-9, 14-32, 42, 43 limited to SEQ ID NOs: 1, 3, 4, 5, 8

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/30627

----continuation of Box III (Lack of Unity of Invention)----

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I+: Claims 1-32, 42, 43 drawn to a fusion protein composition comprising signal peptide, an elafin region, a linker and a Fc region.

The fusion protein will be searched to the extent that the the elafin region encompasses SEQ ID NO: 1; the linker encompasses SEQ ID NO: 3; the Fc region encompasses SEQ ID NO: 4; the fusion protein encompasses SEQ ID NO: 5; and the polynucleotide encoding the fusion protein encompasses SEQ ID NO: 8. It is believed that claims 1-2, 7-9, 14-32, 42, 43 read on this first named invention and thus these claims will be searched without fee to the extent that they encompass a fusion protein comprising SEQ ID NOs: 1, 3-5, 8. Additional fusion protein(s) will be searched upon payment of additional fees. Applicant must specify the claims that encompass any additional elected fuson protein(s). Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be: a fusion protein comprising elafin region SEQ ID NO: 1 having V5G, V9G, M25L mutations; linker of SEQ ID NO: 3; Fc region of SEQ ID NO: 4, fusion protein SEQ ID NO: 6 and polynucleotide SEQ ID NO: 9 (claims 1-9, 14-32, 42, 43).

[Note, see instant Specification, pg 46, "INFORMAL SEQUENCE LISTING", SEQ ID NO: 5 represents: "Variant 2 protein sequence; Native form-without any mutation"; SEQ ID NO: 6 represents: "Variant 3 protein sequence, wherein the elafin region comprises mutations VSG, V9G, M25L, and M51V relative to SEQ ID NO:l"].

Group II: Claims 33-41, drawn to a method of producing a fusion protein.

Group III: Claims 44-46, drawn to a method of treating an inflammatory disease.

The inventions listed as Groups I+, II, and III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features:

Group I+ has the special technical feature of a composition comprising a fusion protein comprising signal peptide, an elafin region, a linker and a Fc region, not required by Groups II or III.

Group II has the special technical feature of method steps involved in making a fusion protein, not required by Groups I+ or III.

Group III has the special technical feature of a method of treating an inflammatory disease, not required by Groups I+ or II.

Among the inventions listed as Groups I+ is the specific elafin-Fc fusion proteins, recited therein. The inventions do not share a special technical feature, because no significant structural similarities can readily be ascertained among elafin-Fc fusion proteins.

Common Technical Feature:

The inventions of Groups I-III share the technical features of a fusion protein comprising a signal peptide, an elafin region, a linker, and a Fc region; and a polyncuIeotide, a recombinant vector and a recombinant cell, comprising the same.

However, said common technical features do not represent a contribution over the prior art, and is anticipated by US 2013/0011399 A1 to Timmer et al. (hereinafter "Timmer").

As to claim 1, Timmer teaches a fusion protein comprising a signal peptide, an elafin region, a linker, and a Fc region (claim 3; "An isolated fusion protein comprising at least one human Elafin polypeptide operably linked to a second polypeptide, wherein the second polypeptide comprises at least one of the following: an immunoglobulin Fc polypeptide"; claim 14: "14. The isolated fusion protein of claim 3, wherein the Elafin polypeptide and the immunoglobulin Fc polypeptide are operably linked via a hinge region, a linker region, or both a hinge region and linker region"; para [0153]; containing a mammalian secretion signal sequence up stream of the SLPI or Elafin gene insertion site.).

As to claim 16, Timmer teaches a polynucleotide encoding the fusion protein of claim 1 (para [0153]; The genes encoding human SLPI and Elafin were PCR amplified from human spleen cDNA (Zyagen). Specific point mutations within the gene encoding SLPI, Elafin or the Fc region were generated by overlapping PCR. The SLPI or Elafin encoding gene was cloned in frame with a gene encoding the hinge region, followed by a CH2 domain, and a CH3 domain of human IgG1, IgG2, IgG3, IgG4, or IgM into a mammalian expression vector, containing a mammalian secretion signal sequence up stream of the SLPI or Elafin gene insertion site. In some cases, these vectors were further modified, wherein the gene encoding a linker sequence and either SLPI or Elafin was cloned in frame to the 3' end of the CH3 domain, to generate SLPI-Fc-SLPI, Elafin-Fc-Elafin, SLPI-Fc-Elafin, or Elafin-Fc-SLPI").

Timmer further teaches a recombinant vector (para [0153], as discussed above) and a recombinant cell (para [0153]; "These expression vectors were transfected into mammalian cells (specifically HEK293 or CHO cells)")

-----continued on next sheet-----

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/30627

------continued from previous sheet------

As to claim 42, Timmer teaches a composition comprising the fusion protein of claim 1 (claims 3, 14 and para [0153]).

As the common technical features were known in the art at the time of the invention, they cannot be considered common special technical features that would otherwise unify the groups. The inventions lack unity with one another.

Therefore, Groups I+, II and III lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.