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(54) **Title:** DRY POWDER FORMULATIONS OF DNASE I

(57) **Abstract:** DNase I formulations for pulmonary administration and, more particularly, but not exclusively, a dry powder formulation comprising, as an active ingredient, human DNase I, methods, dry powder inhalation devices and systems for the therapeutic use thereof are provided.

DRY POWDER FORMULATIONS OF DNASE I

FIELD AND BACKGROUND OF THE INVENTION

5 The present invention, in some embodiments thereof, relates to DNase I formulations for pulmonary administration and, more particularly, but not exclusively, to a dry powder formulation comprising, as an active ingredient, human DNase I, methods, dry powder inhalation devices and systems for the therapeutic use thereof.

 Human DNase I is a member of the mammalian DNase I family (EC 3.1.21.1).
10 DNase I belongs to the class of Mg^{2+} and Ca^{2+} dependent endonucleases, whose hydrolytic activity depends on the presence of bivalent metals. Magnesium ion is involved in electrophilic catalysis of the phosphodiester bond cleavage, whereas Ca^{2+} maintains optimal enzyme conformation. DNase I cleaves DNA preferentially at phosphodiester linkages adjacent to a pyrimidine nucleotide, yielding 5'-phosphate-
15 terminated polynucleotides with a free hydroxyl group on position 3', on average producing tetranucleotides. It acts on single-stranded DNA, double-stranded DNA, and chromatin.

 The principal therapeutic use of human DNase has been to reduce the viscoelasticity of pulmonary secretions (including mucus) in such diseases as
20 pneumonia and cystic fibrosis (CF), thereby aiding in the clearing of respiratory airways. Mucus also contributes to the morbidity of chronic bronchitis, asthmatic bronchitis, bronchiectasis, emphysema, acute and chronic sinusitis, and even the common cold. DNase I is effective in reducing the viscoelasticity of pulmonary secretions and fluids by hydrolyzing high-molecular-weight DNA present in pulmonary
25 secretions and fluids. DNase has also been proposed for non-pulmonary disorders, for example, treatment of male infertility and uterine disorders (see US 2007/0259367), inhibition of metastatic growth (see US 7,612,032) and for treatment of sepsis and viral, bacterial, fungal and protozoan infections.

 DNA encoding human DNase I was isolated and sequenced, and expressed in
30 recombinant host cells, thereby enabling the production of human DNase in commercially useful quantities. Recombinant human DNase (rhDNase) (e.g. dornase alfa; Pulmozyme®, Genentech., CA), expressed in Chinese hamster ovary (CHO) cells, has been found to be clinically effective for CF.

Recombinant human DNase (rhDNase) (e.g. dornase alfa; Pulmozyme®, Genentech., CA) was approved for clinical use by the FDA in 1994, and additional human clinical trials with recombinant human DNase I are currently in progress for CF (see NCT01155752; NCT0017998; NCT00117208 and NCT00204685) and other
5 chronic, respiratory diseases such as atelectasis (see NCT01095276 and NCT00671323) and Sjogren's Syndrome. In general, safety and efficacy of the rhDNase has been demonstrated. Pulmozyme® is provided as a liquid protein formulation ready for use in nebulizer systems.

In addition to nebulizer systems, pulmonary administration of drugs and other
10 pharmaceuticals can be accomplished by provision of a dry powder formulation for inhalation by means of suitable inhalers known as dry powder inhalers (DPIs).

SUMMARY OF THE INVENTION

According to an aspect of some embodiments of the present invention there is provided an inhalable dry powder formulation comprising a human DNase I protein and
15 particles of a physiologically acceptable pharmacologically-inert solid carrier.

According to an aspect of some embodiments of the present invention there is provided a dry powder inhaler device, comprising the inhalable dry powder formulation comprising a human DNase I protein and particles of a physiologically acceptable pharmacologically-inert solid carrier a means for introducing the inhalable dry powder
20 formulation into the airways of a subject by inhalation.

According to some embodiments of the present invention the device is a single dose or a multidose inhaler.

According to some embodiments of the present invention the device is pre-metered or device-metered.

25 According to some embodiments of the present invention the human DNase I protein is a recombinant human DNase I protein.

According to some embodiments of the present invention the human DNase I protein is a plant-expressed human DNase I protein.

30 According to some embodiments of the present invention the human DNase I protein comprises an N-terminal Glycine residue.

According to some embodiments of the present invention the human DNase I protein comprises the amino acid sequence as set forth in SEQ ID NO: 6.

According to some embodiments of the present invention the human DNase I protein comprises the amino acid sequence as set forth in SEQ ID NO:5.

According to some embodiments of the present invention the human DNase I protein has at least one core xylose and at least one core α -(1,3) fucose.

5 According to some embodiments of the present invention the human DNase I protein has reduced susceptibility to actin inhibition of endonuclease activity as compared with that of mammalian cell expressed human recombinant DNase I.

According to some embodiments of the present invention the carrier is selected from the group consisting of (a) at least one crystalline sugar selected from the group consisting of glucose, arabinose, maltose, saccharose, dextrose, and lactose; and (b) at least one polyalcohol selected from the group consisting of mannitol, maltitol, lactitol, and sorbitol.

According to some embodiments of the present invention the carrier is in a form of finely divided particles having a mass median diameter (MMD) in the range of 0.5 to 15 10 microns.

According to some embodiments of the present invention the carrier is in a form of finely divided particles having a mass median diameter (MMD) in the range of 1.0 to 6.0 microns.

According to some embodiments of the present invention the carrier is in a form of coarse particles having a mass diameter of 50- 500 microns.

According to some embodiments of the present invention the coarse particles have a mass diameter of 150 microns to 400 microns.

According to some embodiments of the present invention the carrier comprises a mixture of coarse particles having a mass diameter of 150 microns to 400 micron and 25 finely divided particles having a MMD in the range of 0.5- 10 microns.

According to some embodiments of the present invention the dry powder formulation further comprises, as an active ingredient, a magnesium salt.

According to some embodiments of the present invention the dry powder formulation further comprises, as an active ingredient, an agent for inhibiting formation 30 of G actin and/or enhancing formation of F actin.

According to some embodiments of the present invention the dry powder formulation further comprises one or more additive materials selected from the group

consisting of an amino acid, a water soluble surface active agent, a lubricant, and a glidant.

According to some embodiments of the present invention the human DNase I protein is at least 90-95% pure human DNase I protein.

5 According to some embodiments of the present invention the inhalable pharmaceutical composition further comprising plant beta-acetylhexosaminidase enzyme protein. In some embodiments the plant beta-acetylhexosaminidase enzyme protein is inactivated beta-acetylhexosaminidase enzyme protein. In some
10 embodiments the beta-acetylhexosaminidase enzyme protein is heat inactivated beta-acetylhexosaminidase enzyme protein.

According to some embodiments of the present invention the human DNase I is in association with diketopiperazine.

According to some embodiments of the present invention the diketopiperazine is selected from the group consisting of succinyl diketopiperazine, glutaryl
15 diketopiperazine, maleyl diketopiperazine, and fumaryl diketopiperazine or a pharmaceutically acceptable salt thereof.

According to an aspect of some embodiments of the present invention there is provided a method of reducing extracellular DNA in a subject in need thereof, the method comprising administering to the subject in need thereof an effective amount of
20 the inhalable dry powder formulation comprising human DNase I of the invention.

According to some embodiments of the present invention the subject is suffering from a disease or condition selected from the group consisting of male infertility, metastatic cancer, a viral, bacterial, fungal or protozoan infection, sepsis, atherosclerosis, diabetes, delayed type hypersensitivity and a uterine disorder.

25 According to an aspect of some embodiments of the present invention there is provided a method for reducing DNA in a pulmonary secretion or fluid of a subject in need thereof, the method comprising administering to the subject in need thereof an effective amount of the inhalable dry powder formulation comprising human DNase I of the invention.

30 According to an aspect of some embodiments of the present invention there is provided a method for the prevention and/or treatment of a pulmonary disease or condition associated with excess DNA in a pulmonary secretion in a subject in need

thereof, the method comprising administering to a subject in need thereof an effective amount of the inhalable dry powder formulation comprising human DNase I of the invention.

According to some embodiments of the present invention the subject is suffering
5 from a respiratory disease selected from the group consisting of acute or chronic bronchopulmonary disease, atelectasis due to tracheal or bronchial impaction, and complications of tracheostomy.

According to some embodiments of the present invention the acute or chronic
10 bronchopulmonary disease is selected from the group consisting of infectious pneumonia, bronchitis or tracheobronchitis, bronchiectasis, cystic fibrosis, asthma, chronic obstructive pulmonary disease (COPD), TB or fungal infections.

According to some embodiments of the present invention the effective amount
of the dry powder formulation is a single dose of 0.1 to 25 mg DNase I, administered
daily.

15 According to some embodiments of the present invention the effective amount of the dry powder formulation is a single dose of 0.5 to 15 mg DNase I, administered daily.

According to some embodiments of the present invention the effective amount
of the dry powder formulation is a single dose of 1.0 to 10 mg DNase I, administered
20 daily.

According to some embodiments of the present invention the effective amount
of the dry powder formulation is a single dose of 2.0 to 5 mg DNase I, administered
daily.

25 According to some embodiments of the present invention the effective amount of the dry powder formulation is a single dose of 2.0- 3.0 mg DNase I, administered daily.

According to some embodiments of the present invention the effective amount
of the dry powder formulation is a plurality of doses, each dose comprising 1.0-3.0 mg
DNase, the doses administered at least twice, 2-3 times, 2-4 times or 2-6 times daily.

30 According to some embodiments of the present invention the effective amount of the dry powder formulation is a plurality of doses, each dose comprising 1.0-3.0 mg DNase, the doses administered once every 36 hours, once every 36-48 hours, once

every 36-72 hours, once every 2-3 days, once every 2-4 days, once every 2-5 days, or once every week.

According to some embodiments of the present invention the effective amount of the dry powder formulation is a plurality of doses, each dose comprising 1.0-3.0 mg DNase, the doses administered once every 36 hours, once every 36-48 hours, once every 36-72 hours, once every 2-3 days, once every 2-4 days, once every 2-5 days, or once every week.

Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the invention, exemplary methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

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BRIEF DESCRIPTION OF THE DRAWINGS

Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the invention. In this regard, the description taken with the drawings makes apparent to those skilled in the art how embodiments of the invention may be practiced.

In the drawings:

FIG. 1 is an amino acid sequence alignment of the plant recombinant human DNase I (prhDNase I) encoded by the nucleic acid of the invention [SEQ ID NO: 1, sequence (b)], and native human DNase I protein [(GenBank: NM_005223, sequence (a)](SEQ ID NO: 2), including the native signal leader peptide (highlighted in red, SEQ ID NO: 3). The Arabidopsis ABPI endoplasmic reticulum targeting signal peptide (SEQ ID NO: 4) is highlighted in green;

FIG. 2 is a graph showing the results of targeting expression of recombinant human DNase I to different plant organelles. rhDNase I was expressed in whole tobacco plants and targeted for secretion (to the apoplast) via an N-terminal ER targeting signal

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peptide (APO), targeted to the vacuole, via an N-terminal ER targeting signal and a C-terminal vacuolar targeting signal peptide (VAC) and targeted to the endoplasmic reticulum via an N-terminal ER targeting signal peptide and a C-terminal ER retention signal peptide (ER). Activity of rhDNase I in the cells of the tobacco plant was monitored by immunoreactivity (ELISA) and according to catalytic activity (DNA-methyl green assay), adjusted for amount of biomass in sample and expressed as phrDNase I relative to expression in apoplast-targeted cells (100%). Note the significantly greater yield when the rhDNase I is targeted to the apoplast;

FIG. 3 shows an SDS-PAGE gel illustrating the size and purity of plant-expressed rh DNase I. 1.25 μ g, 2.5 μ g, 5 μ g and 10 μ g of commercial human DNase (Pulmozyme®) (lanes 1-4, respectively) or purified prh DNase I (lanes 6-9, respectively) were separated on 15% Tris-Glycine SDS-PAGE, stained with Coomassie blue, and analyzed in comparison to molecular weight standards (lanes 5 and 10). Note the single, predominant band of the plant-expressed enzyme migrating slightly but discernibly faster than that of commercial human DNase (Pulmozyme®), and the absence of detectable protein impurities;

FIG. 4 shows an SDS-PAGE gel illustrating the size, purity and antigenic identity of plant-expressed rh DNase I. 10 ng, 50 ng, 100 ng and 200 ng of commercial human DNase (Pulmozyme®) (lanes 1-4, respectively) or purified prh DNase I (lanes 6-9, respectively) were separated on 15% Tris-Glycine SDS-PAGE, transferred to nitrocellulose membrane, probed with whole antiserum primary antibody obtained from rabbits immunized against Pulmozyme®, and visualized with secondary goat anti-rabbit IgG HRP-conjugated antibody. Note the single, predominant band of the immunoreactive, plant-expressed enzyme migrating and reacting similarly to that of commercial human DNase (Pulmozyme®), and the absence of detectable protein impurities;

FIGs. 5A-5D shows an IEF gel and capillary electropherogram of human DNase (Pulmozyme®) and purified, plant expressed rh DNase I. Figure 5A shows the resolution of 6 μ g of commercial human DNase (Pulmozyme®) (lane 1) or 6 μ g of purified, plant expressed rh DNase I (lanes 2-3) on an isoelectric focusing gel. Figure 5B shows the resolution of 8 μ g of commercial human DNase (Pulmozyme®) (lane 3) or 8 μ g of purified, plant expressed rh DNase I from a different line of BY2 cells expressing

the prhDNase I (lane 2) on an isoelectric focusing gel. Note that Pulmozyme® resolves into multiple bands with isoelectric points (pi) between pi 3.5 and pi 4.5 in a pH gradient of pH 3 to pH 7, while in both of the purified, plant expressed rh DNase I preparations the DNase I resolved at an isoelectric point (pi) between pi 4.2 and pi 4.5, in two major bands and one minor band. Electrophoresis conditions included 100mV-1 hour, 200mV-1 hour and 500mV-1.5 hours. Lanes 4 (Figure 5A) and lane 1 (Figure 5B) contain protein standards for comparison. Figure 5D is an electropherogram of image capillary isoelectric focusing (scale = pi 3.3-6.1) analysis of purified, plant expressed rh DNase I, showing the resolution of the prh DNase I as observed in the IEF gel [one major peak (3) at pi 4.41 and two minor peaks (4 and 5) at pi 4.27 and 4.21]. 1 and 2 are pi markers 5.85 and 3.59. Figure 5C is an electropherogram of the blank scale with pi markers 1 and 2;

FIGs. 6A-6B show the molecular mass analysis of purified, plant expressed rh DNase I by mass spectrometry using 2.5 micrograms of the purified prh DNase I using a matrix-assisted laser desorption ionization time-of-flight (MALDI-ToF) mass spectrometer, with sinapinic acid as a matrix. Figure 6A showing the entire spectrum from 20000 to 180000 m/z and Figure 6B showing an enlarged segment of the prh-DNaseI peak at about 32000 m/z;

FIG. 7 is a figure representing a putative amino acid sequence of the purified, plant expressed rh DNase I (SEQ ID NO: 5), derived from the sequences of overlapping peptides produced by partial proteolytic digestion (see SEQ ID NOs: 17-276) and separated and analyzed on RP-HPLC and mass spectrometry. Unconfirmed amino acids are marked in red, glycosylation sites are underlined and the N-terminal Glycine residue is marked in green;

FIGs. 8A-8C represent analysis of the glycan structure of the purified, plant expressed rh DNase I, as determined by exoglycosidase digestion and NP-HPLC analysis of the resulting de-glycosylated polypeptides. FIG. 8A is an NP-HPLC profile of total glycans released following PNGase A digestion of the glycosylated prhDNase I, derived from three separate exemplary batches of prhDNase I. The numbers above the discrete peaks correspond to specific glycan structures illustrated in FIG. 8B. FIG. 8B is a chart showing individual glycan structures released from prhDNase I by PNGase A digestion, and their relative abundance (in percentages) from total released glycans, for

each of the three exemplary batches tested. Note the predominance of two main glycan peaks representing a high percentage of glycan structures (over 80%) containing mannose 3- β -(1,2) xylose- α -(1,3) fucose [Fc(3)M3X] and/or mannose 4- α -(1,2) xylose. FIG. 8C shows an NP-HPLC profile of total glycans released from prhDNase I using PNGase A endoglycosidase (top) compared to the profile of N-linked glycans released from Pulmozyme® using PNGase F endoglycosidase (specific to high mannose glycans) showing a multitude of glycan variations. The major peaks are annotated with corresponding glycan structures. Note the wide variation in glycosylation pattern found in Pulmozyme®, due to the abundance of bi- and tri-antennary glycans, and sialic acid residues.

FIGs. 9A-9D are chromatograms of RP-HPLC of purified, plant expressed rh DNase I, analyzed at 214 nm (FIG. 9A, 9B) and 280 nm (FIG. 9C, 9D), indicating a preparation of the prh DNase I having less than 7% impurities (93.77% pure measured at 214nm, and 93.62% pure measured at 280 nm). Insets (FIGs. 9B and 9D) are expanded views of the prh DNase I peak at 214 nm and 280 nm, respectively;

FIGs. 10A-10B are graphs representing substrate inhibition kinetic plots of purified prh DNase I (open circles O) and commercial human DNase (Pulmozyme®) (closed circles ·), using the DNaseAlert™ fluorometric substrate detected at 535 nm and 565 nm. FIG. 10A is a plot of the initial velocity as a function of substrate concentration. Note the superior kinetics (greater $V_{m_{\max}}$, lower K_M , e.g. higher specific activity) of the purified prh DNase I. FIG. 10B is a double reciprocal plot of initial velocity as a function of substrate concentration. K_M and V_{\max} values were calculated using the following linear regression equations (plotted at substrate concentration of 2-15 μ M) and R^2 values: prh DNase I : $y = 41.377x + 1.8693$, $R^2 = 0.9894$; Pulmozyme®: $y = 120.17x + 4.8316$, $R^2 = 0.9921$, indicating substrate-inhibition-like kinetics for both prh DNase I and Pulmozyme®;

FIG. 11 is an activity plot of purified, plant expressed rh DNase I (open circles O) and commercial human DNase (Pulmozyme®) (closed circles ·), illustrating resistance to actin inhibition of DNase I activity. AOD (620 nm) is plotted as a function of G-actin concentration, using the Methyl Green substrate, to yield IC_{50} values. Note the reduced susceptibility to G-actin inhibition of the purified prh DNase I, compared to that of commercial human DNase (Pulmozyme®);

FIGs. 12A-D are histograms illustrating the effect of increasing concentrations of DNase I on the elastic modulus of sputum from Cystic Fibrosis patients, determined using time-sweep measurements with a rheometer (HAAKE RheoStress I, Thermo Fisher Scientific GmbH, Germany). FIG. 12A represents a comparison of the effect of purified, plant expressed rh DNase I (dark bars) and commercial human DNase (Pulmozyme®) (hatched bars) on the elastic modulus of CF patient sputa, measured at 2, 10 and 20 μg DNase I /gr sputum. DNA content of the sputum was 4.66 μg DNA/ gr sputum. Each value represents at least 2 determinations measured from each sputum sample. FIGs. 12B-12D show the effect of prhDNase I on the elastic modulus of sputa collected from individual patients. Note the pronounced, superior and consistent, dose-dependent reduction of sputum elastic modulus following incubation with prh DNase I, as compared to the Pulmozyme®;

FIGs. 13A-D are histograms illustrating the effect of increasing concentrations of DNase I on the viscous modulus of sputum from Cystic Fibrosis patients, measured using time-sweep technique with a rheometer (HAAKE RheoStress I, Thermo Fisher Scientific GmbH, Germany). FIG. 13A represents a comparison of the effect of purified, plant expressed rh DNase I (dark bars) and commercial human DNase (Pulmozyme®) (hatched bars) on the viscous modulus of CF patient sputa, measured at 2, 10 and 20 μg DNase I /gr sputum. DNA content of the sputum was 4.66 μg DNA/ gr sputum. Each value represents at least 2 determinations measured from each sputum sample. FIGs. 13B-13D shows the effect of prhDNase I on the viscous modulus of sputa collected from individual patients. Note the pronounced, superior and consistent, dose-dependent reduction of sputum viscous modulus following incubation with prh DNase I, as compared to the Pulmozyme®;

FIGs. 14A and 14B are histograms representing the effect of prhDNase I on the rheological properties of sputum from Cystic Fibrosis patients, measured using time sweep technique, expressed as percent change rheological properties (elastic modulus, FIG. 14A and viscous modulus, FIG. 14B) compared to an untreated sample. FIG. 14A shows the percent change of elastic modulus of the sputa of 4 individual patients (black bars= untreated control samples; hatched bars= 2 μg prhDNase I; gray bars= 20 μg prhDNase I). FIG. 14B shows the percent change of viscous modulus of the sputa of 4 individual patients (black bars= untreated control samples; hatched bars= 2 μg

prhDNase I; gray bars= 20 μ g prhDNase I). Note the consistent pronounced reduction in rheological properties following incubation of the sputa with prh DNase I;

FIG. 15 is a histogram illustrating the synergic effect of purified, plant expressed rh DNase I on the reduction of CF sputum elastic modulus by Magnesium (Mg^{2+}) (0, 25, 50 and 100 mM), measured using time sweep technique, with rheometer (HAAKE RheoStress I, Thermo Fisher Scientific GmbH, Germany). Elastic modulus of the sputum was determined at indicated concentrations of Mg^{2+} (mM Mg^{2+}) in the presence (cross-hatched bars) or absence (dark bars, buffer only) of prh DNase I (2 μ g prh DNase I /gr sputum). Each value represents at least 2 determinations. Note the synergic reduction of sputum elastic modulus following incubation with the prh DNase I and Magnesium, as compared to Magnesium alone;

FIG. 16 is a histogram illustrating the effect of Magnesium salts on DNase I reduction of CF sputum viscous modulus. Figure 16 shows the synergic effect of purified, plant expressed rh DNase I on the reduction of CF sputum viscous modulus by Magnesium ($MgCl_2$) (0, 25, 50 and 100 mM), measured with a rheometer (HAAKE RheoStress I, Thermo Fisher Scientific GmbH, Germany). Viscous modulus of the sputum was determined at the indicated concentrations of Mg^{2+} (mM Mg^{2+}) in the presence (cross hatched bars) or absence (grey bars, buffer only) of prh DNase I (2 μ g DNase I /gr sputum). Each value represents at least 2 determinations. Note the synergic reduction of sputum viscous modulus following incubation with the prh DNase I and Magnesium, as compared to Magnesium alone;

FIG. 17 is a graph illustrating the effect of different Magnesium salts on the DNase catalytic activity of purified, plant expressed rh DNase I. Catalytic activity of rh DNase I was measured in the presence of increasing concentrations (0.5-100 mM) of Magnesium chloride ($MgCl_2$, closed circles \bullet) or Magnesium sulphate ($MgSO_4$, open diamonds \diamond), using the methyl green substrate and expressed as the change in absorbance at 630 nm (Δ OD 630 nm) after 3 hours incubation with the enzyme. Note that neither magnesium salt inhibited rh DNase I activity up to 50 mM, and that rh DNase I activity was only slightly impaired by $MgSO_4$ at 100 mM.

FIGs. 18A-18D are histograms illustrating the effect of increasing concentrations of DNase I on the rheological properties of sputum from Cystic Fibrosis patients, determined using the stress sweep technique with a rheometer (HAAKE RheoStress I,

Thermo Fisher Scientific GmbH, Germany), expressed as the stress (Pa) at cross-over of elastic and viscous modules (phase angle = 45°). FIGs. 18A-18D represents a comparison of the effect of purified, plant expressed rh DNase I (dark bars) and commercial human DNase (Pulmozyme®) (hatched bars) on the rheological properties of four individual CF patient sputa, measured at 0.2, 2 and 20 µg DNase I /gr sputum. DNA content of the sputum was 3.09 mg DNA/ gr sputum, 3.15 mg DNA/ gr sputum, 8.36 mg DNA/ gr sputum and 3.89 mg DNA/ gr sputum (FIGs. 18A-18D, respective). Each value represents at least 2 determinations measured from each sputum sample. Note the pronounced and consistent, dose-dependent reduction in cross-over stress values following incubation with prh DNase I;

FIGs. 19A to 19D are histograms representing the effect of prhDNase I and Pulmozyme® on the rheological properties of sputum from four different Cystic Fibrosis patients (19A-19D), measured using stress sweep technique, expressed as the stress (Pa) at cross-over of elastic and viscous modules (phase angle = 45°). DNA content of the sputum was 2.16 mg DNA/ gr sputum, 2.63 mg DNA/ gr sputum, 3.45 mg DNA/ gr sputum and 4.17 mg DNA/ gr sputum (FIGs. 19A-19D, respective) Black bars= prhDNase I; hatched bars= Pulmozyme®. DNase I concentration (0, 2 or 20 µg DNase I/ gr sputum). Note the consistent pronounced reduction in rheological properties following incubation of the sputa with prh DNase I, significantly outperforming Pulmozyme® in 3 out of the four patients;

FIGs. 20A-20C are histograms illustrating the effect of Magnesium salts and DNase I on the rheological properties of CF sputum samples from three different Cystic Fibrosis patients, measured using the stress sweep technique with a rheometer (HAAKE RheoStress I, Thermo Fisher Scientific GmbH, Germany), expressed as the stress (Pa) at cross-over of elastic and viscous modules (phase angle = 45°). FIG. 20A-patient A (1.84 mgDNA/g sputum), FIG. 20B- patient B (3.46 mgDNA/g sputum) and FIG. 20C (2.39 mg DNA/g sputum). Dark columns are measurements without Magnesium, grey columns are represent measurement with 100 mM MgSO₄, measured at 0 (control) 2 and 20 µg DNase I /gr sputum. Each value represents at least 2 determinations measured from each sputum sample. Note the pronounced and synergistic disruption of sputum elastic structure following incubation with prh DNase I and MgSO₄.

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to dry powder formulations of human DNase I as an active ingredient, suitable for administration by inhalation by means of a dry powder inhaler (DPI), processes for the preparation of such a formulation, and methods of using such a formulation for reducing extracellular DNA in a subject in need thereof and for the prevention and/or treatment of a wide range of conditions including respiratory disorders such as cystic fibrosis (CF), asthma and chronic obstructive pulmonary disease (COPD), as well as other, non-respiratory disorders.

It is to be understood that the invention is not necessarily limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways.

Clinically approved, commercially available recombinant human DNase I (rh DNase I) (dornase alpha®, Pulmozyme ®; Genentech) is supplied as a liquid protein formulation for use in pulmonary administration by inhalation using a nebulizer. Such a liquid formulation is unsuitable for use in other forms and systems for pulmonary administration of drugs, such as dry powder inhalation. The present inventors have devised a dry powder formulation comprising human DNase I, suitable for pulmonary administration and capable of providing catalytically active human DNase I for nucleolytic digestion of extracellular DNA found in secretions, fluids and tissues of the airways, as well as fluids, secretions and tissues accessible via systemic circulation. Surprisingly, such dry powder formulations, typically noted for their efficacy in delivering pharmaceutical compositions to the respiratory lining and alveolar cells, have been found effective for providing catalytically active human DNase I protein to the sputum and other pulmonary secretions, resulting in reduction in the DNA content and improving the rheological properties of the sputum and other secretions. Yet further, when comprising a plant expressed recombinant human DNase I, the efficacy of the dry powder formulation of the invention in treating pulmonary secretions can be enhanced due to the surprisingly favorable kinetic properties and resistance of the prh DNase I to actin inhibition, thereby enhancing the therapeutic value of the human DNase I when provided in such a dry powder formulation.

According to one aspect of some embodiments of the present invention there is provided a pharmaceutical formulation in the form of inhalable dry powder, comprising, as active ingredient a human DNase I polypeptide and particles of a physiologically acceptable pharmacologically-inert solid carrier.

5 As used herein the term "dry powder" refers to a fine particulate composition, with particles of mean mass diameter selected capable of being borne by a stream of air or gas, the dry powder not being suspended or dissolved in a propellant, carrier or other liquid. "Dry powder" does not necessarily imply the complete absence of water molecules from the formulation.

10 As used herein, the term "physiologically inert... carrier" refers to a carrier whose administration to the subject does not result in a physiological response or reaction associated with the response or reaction of the subject to the active ingredient, in this case, the DNase I. In some embodiments, the "physiologically inert... carrier" is devoid of any effect on the pharmaceutical activity of the active ingredient, e.g. the
15 DNase I.

In the field of dry powders for inhalation it is customary to formulate pharmaceutically active substances with carrier particles of inert material such as lactose. The carrier particles are designed such that they have a larger mean median diameter (MMD) than the active substance particles making them easier to handle and
20 store. The smaller active agent particles are bound to the surface of carrier particles during storage, but are torn from the carrier particles upon actuation of the device. This process is often referred to as de-agglomeration. In order to assist in the de-agglomeration it has been proposed to employ so-called force-controlling agents or anti-adherent additives in admixture with active and carrier particles. Force controlling
25 agents can be surface active materials. One such force-controlling agent is magnesium stearate. Dry powder formulations employing magnesium stearate and the like are described in U.S. Pat. No. 6,645,466, which is hereby incorporated by reference, and devices containing powders disclosed in this patent represent particularly preferred
30 embodiments of the present invention. Other force-controlling agents or anti-adherent additives are described in U.S. Pat. No. 6,521,260, which is herein incorporated by reference.

In some embodiments, the inhalable dry powder formulation comprises a carrier comprising a crystalline sugar selected from the group consisting of glucose, arabinose, maltose, saccharose, dextrose and lactose; and at least one polyalcohol selected from the group consisting of mannitol, maltitol, lactitol and sorbitol. In one embodiment, the crystalline sugar is lactose.

In some embodiments, the inhalable dry powder formulation comprises a carrier in the form of finely divided particles, coarse particles or a combination of fine and coarse particles. As used herein, the term "finely divided particles" includes particles of generally 0.5 to 100 microns in diameter, in the range of 1- 50 microns mass median diameter (MMD), in the range of 1- 25 microns mass median diameter (MMD), in the range of 1-6 microns mass median diameter (MMD), 0.1-0.5 microns mass median diameter (MMD), 0.5-1.0 microns mass median diameter (MMD), 1.0-2.0 microns mass median diameter (MMD), 2.0- 5 microns mass median diameter (MMD). As used herein, the term "coarse particles" includes particles of generally greater than 50 microns in diameter, in the range of 50- 500 microns mass median diameter (MMD), in the range of 150-400 microns MMD. Various embodiments will entail more specific size ranges. The finely divided particles can be assemblages of crystalline plates, with the active agents entrapped or coated onto the crystalline surfaces of the particle. The particles can also be spherical shells or collapsed spherical shells with the active agent dispersed throughout. Such particles can be obtained by spray drying a co-solution of a co-composition (such as diketopiperazine, as described hereinbelow) and the active agent. Other forms of particles are contemplated and encompassed by the term.

The dry powder formulation may comprise an adsorption enhancer.

The enhancer used can be any of a number of compounds which act to enhance absorption through the layer of epithelial cells lining the alveoli of the lung, and into the adjacent pulmonary vasculature. The enhancer can accomplish this by any of several possible mechanisms:

- (1) Enhancement of the paracellular permeability of DNase by inducing structural changes in the tight junctions between the epithelial cells;
- (2) Enhancement of the transcellular permeability of DNase by interacting with or extracting protein or lipid constituents of the membrane, and thereby perturbing the membrane's integrity;

(3) Interaction between enhancer and DNase which increases the solubility of DNase in aqueous solution. This may occur by preventing formation of DNase aggregates, or by solubilizing DNase molecules in enhancer micelles;

(4) Decreasing the viscosity of, or dissolving, the mucus barrier lining the alveoli and passages of the lung, thereby exposing the epithelial surface for direct
5 absorption of the DNase.

Enhancers may function by only a single mechanism set forth above, or by two or more. An enhancer which acts by several mechanisms is more likely to promote efficient absorption of DNase than one which employs only one or two. For example,
10 surfactants are a class of enhancers which are believed to act by all four mechanisms listed above. Surfactants are amphiphilic molecules having both a lipophilic and a hydrophilic moiety, with varying balance between these two characteristics. If the molecule is very lipophilic, the low solubility of the substance in water may limit its usefulness. If the hydrophilic part overwhelmingly dominates, however, the surface
15 active properties of the molecule may be minimal. To be effective, therefore, the surfactant must strike an appropriate balance between sufficient solubility and sufficient surface activity.

In some embodiments of the invention the dry powder formulation the human DNase I is associated with a diketopiperazine, also acting as an adsorption enhancer.
20 Diketopiperazines, in addition to making aerodynamically suitable finely divided particles, also facilitate transport across cell layers, further speeding absorption into the circulation. Diketopiperazines can be formed into particles that incorporate a drug or particles onto which a drug can be adsorbed. The combination of a drug and a diketopiperazine can impart improved drug stability. These particles can be
25 administered by various routes of administration. As dry powders these particles can be delivered by inhalation to specific areas of the respiratory system, depending on particle size. Additionally, the particles can be made small enough for incorporation into an intravenous suspension dosage form. Oral delivery is also possible with the particles incorporated into a suspension, tablets or capsules. Diketopiperazines may
30 also facilitate absorption of the DNase I.

In some embodiments of the present invention, the diketopiperazine is a derivative of 3,6-di(4-aminobutyl)-2,5-diketopiperazine, which can be formed by

(thermal) condensation of the amino acid lysine. Exemplary derivatives include 3,6-di(succinyl-4-aminobutyl)-, 3,6-di(maleyl-4-aminobutyl)-, 3,6-di(glutaryl-4-aminobutyl)-, 3,6-di(malonyl-4-aminobutyl)-, 3,6-di(oxalyl-4-aminobutyl)-, and 3,6-di(fumaryl-4-aminobutyl)-2,5-diketopiperazine. The use of diketopiperazines for drug delivery is known in the art (see for example U.S. Pat. Nos. 5,352,461, 5,503,852, 6,071,497, and 6,331,318", each of which is incorporated herein by reference for all that it teaches regarding diketopiperazines and diketopiperazine-mediated drug delivery). Pulmonary drug delivery using finely divided diketopiperazine particles is disclosed in U.S. Pat. No. 6,428,771, which is hereby incorporated by reference in its entirety.

As used herein the term "human DNase I protein" refers to a human DNase I (deoxyribonuclease I; EC 3.1.21.1; DNase I) polypeptide. Human DNase I is classified as an endonuclease, which cleaves DNA to produce 5' phosphodi- and 5' phosphooligonucleotide end products, with a preference for double stranded DNA substrates and alkaline pH optimum. Other members of the DNase I family of endonucleases are DNase X, DNase lambda, DNASIL2 and tear lipocalin in humans. DNase I is also known, inter alia, as alkaline DNase, bovine pancreatic (bp) DNase, DNase A, DNA phosphatase and DNA endonuclease, for example, in *Bos taurus*. According to some embodiments of the invention, the human DNase I protein is the mature human DNase I protein, having the amino acid sequence as set forth in SEQ ID NO: 6. It will be appreciated that the human DNase I protein can be a modified human DNase I protein, having an amino acid sequence different than that of SEQ ID NO: 6 while maintaining characteristic structure and/or function of DNase I. One non-limiting example of a modified human DNase I protein encoded by the expression vector of the invention is SEQ ID NO: 5.

According to yet other embodiments of the invention, the inhalable dry powder comprises a variant human DNase protein. Variant human DNase proteins, having altered catalytic and/or other biochemical and structural properties, such as altered actin affinity, cofactor requirements, pH optimum, increased shelf life in storage and the like, enhanced recombinant expression or fusion proteins have been disclosed (see, for example, EC 3.1.21.2; EC 3.1.21.3; EC 3.1.21.4; EC 3.1.21.5; EC 3.1.21.6 and EC 3.1.21.7). Suitable modified DNase I polypeptides include, but are

not limited to DNase polypeptides disclosed in US6,348,343; US6,391,607; US7,407,785; US7,297,526 and WO2008/039989.

According to some embodiments, the inhalable dry powder formulation comprises a DNase-like protein polypeptide. The term "DNase I-like protein" refers to an enzyme having an enzyme classification of EC 3.1.21.x, where "x" is a positive integer. As used herein, "DNase I-like proteins" are a subset of "DNases." The amino acid sequences of many DNases, and the coding sequences encoding such DNase proteins are well known in the art, available from public genomic databases such as GenBank, SwissProt, EMBL and many others.

According to yet further embodiments of the invention, the inhalable dry powder formulation comprises a human DNase I protein comprising an N-terminal glycine residue, for example, SEQ ID NO: 5.

According to some embodiments of the invention, the inhalable dry powder formulation comprises a recombinant human DNase I protein. As used herein, the term "recombinant human DNase I protein" refers to a human DNase I protein exogenously produced in a cell transformed with, and expressing an exogenous human DNase I coding sequence. Non-limiting examples of recombinant human DNase I protein are detailed herein, include the mammalian cell-expressed Pulmozyme® and a plant-expressed human recombinant DNase I (SEQ ID NO: 5).

According to some embodiments of the invention, the inhalable dry powder formulation comprises a human DNase I protein comprising the amino acid sequence as set forth in SEQ ID NO: 5, expressed in plant cells. When plant human recombinant DNase I (phr DNase I) was subjected to controlled proteolytic digestion, mass spectrometry of the resulting oligopeptides revealed that the phr DNase I polypeptide could be characterized by a group of overlapping peptide fragments, which together indicated that the full length recombinant human DNase I expressed by the plant cells was identical, in amino acid sequence, to that of native human DNase I, with the addition of a N-terminal glycine residue (see Example 2 and Tables V and Va, hereinbelow). Further analysis of the amino acid sequence confirmed the accuracy of the sequence and identity with the native human DNase I. Thus, according to some embodiments of some aspects of the invention, the inhalable dry powder formulation comprises a phr DNase I comprising a plurality of overlapping DNase I protein peptide

fragments, the fragments having the amino acid sequence as set forth in SEQ ID NOs. 17-276 and 278-291. In some further embodiments, the prh DNase I has an additional N-terminal glycine residue, and in other embodiments, the prh DNase I is devoid of an N-terminal glycine residue.

5 According to some embodiments of some aspects of the invention, the inhalable dry powder formulation comprises a prh DNase I having a molecular mass of about 30 kD, as measured by SDS-PAGE, and about 32 kD, as measured by mass spectrometry. In yet further embodiments, the inhalable dry powder formulation comprises a glycosylated prh DNase polypeptide, comprising a polypeptide moiety having a
10 molecular mass of about 29 kD.

 As shown in Examples 2 and 3, prh DNase I is fully cross-reactive with anti-human recombinant DNase I anti-serum raised against mammalian-cell expressed recombinant human DNase I (e.g. Pulmozyme ®), for example, as detected by Western blotting of the gel-separated plant-expressed recombinant human DNase I (see Figure
15 4) and with ELISA (see Example 3). Thus, according to some embodiments, the inhalable dry powder formulation comprises a prh DNase I protein which is immunoreactive with anti-human DNase I anti-serum.

 In some embodiments the prh DNase I protein is characterized by two major isoforms with isoelectric points between 4.2 and 4.5. In other embodiments, the plant-expressed recombinant human DNase I protein is further characterized by a minor
20 isoform with a p_i between 4.2 and 3.5.

 According to some embodiments of some aspects of the invention, the inhalable dry powder formulation comprises a purified prh DNase I protein, characterized by a purity of at least 85%, at least 87%, at least 90%, at least 91%, at least 91.5%, at least
25 92%, at least 92.5%, at least 93%, at least 93.1%, at least 93.2%, at least 93.3%, at least 93.4%, at least 93.5%, at least 93.6%, at least 93.7%, at least 93.8%, at least 93.9%, at least 94%, at least 94.5%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.1%, at least 99.2%, at least 99.3%, at least 99.4%, at least 99.5%, at least 99.6%, at least 99.7%, at least 99.8%, at least 99.9%, in a range of at least 92.0-
30 99.8% or 100% purity. In some embodiments, purity of the plant-expressed recombinant human DNase I protein is measured by HPLC.

In some embodiments the plant-expressed recombinant human DNase I composition comprises impurities derived from the plant host cell, such as, but not limited to nucleic acids and polynucleotides, amino acids, oligopeptides and polypeptides, glycans and other carbohydrates, lipids and the like. In some 5 embodiments the host-cell derived impurities comprise biologically active molecules, such as enzymes. In other embodiments, the plant-expressed recombinant human DNase I composition comprises plant beta-N-acetylhexosaminidase. Where the host cell is a tobacco cell, or tobacco cell line cell, the plant beta-N-acetylhexosaminidase is a tobacco beta-N-acetylhexosaminidase.

10 In further embodiments the plant beta-N-acetylhexosaminidase is inactivated plant beta-N-acetylhexosaminidase. Inactivation of plant beta-N-acetylhexosaminidase can be effected by physical means, chemical means or biochemical means. Physical inactivation can be performed by heating, freezing, desiccation, etc. Chemical inactivation can be performed by extremes of pH, chemical denaturation, addition or 15 removal of side chains, glycans, amino acids, etc. Biochemical inactivation includes, but is not limited to inhibition by reversible or irreversible inhibitors. Exemplary beta-N-acetylhexosaminidase inhibitors include end-product inhibitors such as N-acetyl-D-glucosamine and beta-methyl-N-acetyl glucosamine, and selective inhibitors such as the compounds disclosed in US Patent Applications US2010016386, US20110237631, 20 US20100087477 and US20120046337. It will be appreciated that preferred methods for inhibition and/or inactivation of the plant beta-N-acetylhexosaminidase are those which also effectively preserve the structural and functional integrity of the plant-expressed human DNase I enzyme.

In some embodiments the plant beta-N-acetylhexosaminidase is inactivated by 25 heating the plant-expressed recombinant human DNase I composition. Suitable temperatures for plant beta-N-acetylhexosaminidase inhibition and/or activation include heating within a range of 37-60 °C for a period of 2 to 5, 10, 20, 30, 40, 50 60 or more minutes. It will be appreciated that effective inhibition and/or inactivation of the plant beta-N-acetylhexosaminidase is achieved more rapidly at higher temperatures and more 30 slowly at lower temperatures of the range. IN some embodiments, the plant-expressed recombinant human DNase I composition is heated in the range of 45-55 °C for 2-10

minutes. In some embodiments, the inhibition/inactivation results in 20, 30, 40, 50, 60, 70, 80% or greater inactivation of the plant beta-N-acetylhexosaminidase.

The present inventors have constructed a plant expression vector for recombinant expression of human DNase I in plant cells comprising a polynucleotide encoding a human DNase I polypeptide, transformed tobacco plants with the vector, and have isolated catalytically active human DNase I from the plants. The biochemical properties of the plant-expressed recombinant human DNase I compare favorably with those of the commercially available clinical standard Pulmozyme® (Dornase alpha), and results with CF sputum suggest an advantageous effect of the plant-expressed recombinant human DNase I on the reduction of rheological parameters of sputum, as well as reduced susceptibility to actin inhibition of the DNase endonuclease activity. Structural differences uncovered between the plant-expressed recombinant human DNase I and mammalian-cell expressed recombinant human DNase I (e.g. Pulmozyme®) (mobility on PAGE, unity of immunoreactive DNase I species as detected on Western blots, see hereinbelow) suggest that both modifications of amino acid sequence and post translational modification resulting from processing of the expressed polypeptide by the plant cell may be responsible for some of the distinguishing functional characteristics of the plant-expressed recombinant enzyme.

Thus, in some embodiments, there is provided an isolated polynucleotide comprising a nucleic acid sequence encoding a human DNase I protein, wherein the human DNase I protein is contiguously linked at the N-terminal to a plant endoplasmic reticulum targeting signal peptide.

According to another aspect of the invention, the human DNase I protein is contiguously linked at the N-terminal to an Arabidopsis ABPI endoplasmic reticulum targeting signal peptide.

As used herein the term "contiguously linked at the N-terminal" refers to covalent attachment of the indicated peptide via a peptide bond to the N-terminal amino acid of the mature protein, for example, human DNase I protein. "Contiguously linked at the C-terminal" refers to covalent attachment of the indicated peptide via a peptide bond to the C-terminal amino acid of the mature protein, for example, human DNase I protein.

As used herein, the term "Arabidopsis ABPI endoplasmic reticulum targeting signal peptide" refers to the leader peptide sequence of the *Arabidopsis thaliana* auxin binding protein, which is capable of directing the expressed protein to the endoplasmic reticulum within the plant cell. In one embodiment, the Arabidopsis ABPI endoplasmic reticulum targeting signal peptide is a 33 amino acid polypeptide as set forth in SEQ ID NO: 4.

Thus, according to another aspect of the present invention, the human DNase I protein contiguously linked at the N-terminal to an Arabidopsis ABPI endoplasmic reticulum targeting signal peptide and the human DNase I protein has an amino acid sequence as set forth in SEQ ID NO: 1.

According to some embodiments of the invention, the human DNase I protein is encoded by a nucleic acid sequence as set forth in SEQ ID NO: 9. According to further embodiments of the invention, the Arabidopsis ABPI endoplasmic reticulum targeting signal peptide is encoded by a nucleic acid sequence as set forth in SEQ ID NO: 10. According to still further embodiments of the invention the human DNase I protein contiguously linked at the N-terminal to an Arabidopsis ABPI endoplasmic reticulum targeting signal peptide is encoded by a nucleic acid sequence as set forth in SEQ ID NO: 12.

In order to express the polypeptide, the sequence encoding same is ligated into a "plant nucleic acid expression construct".

As used herein the term "plant nucleic acid expression construct" refers to a nucleic acid construct which includes the nucleic acid of some embodiments of the invention and at least one promoter for directing transcription of nucleic acid in a host plant cell. Further details of suitable transformation approaches are provided hereinbelow.

According to some embodiments of the invention, there is provided a nucleic acid expression construct comprising the nucleic acid sequence of the invention, and a promoter for directing transcription of the nucleic acid sequence in a plant host cell.

As used herein the term "nucleic acid sequence" refers to a single or double stranded nucleic acid sequence which is isolated and provided in the form of an RNA sequence, a complementary polynucleotide sequence (cDNA), a genomic polynucleotide

sequence and/or a composite polynucleotide sequences (e.g., a combination of the above).

As used herein the phrase "complementary polynucleotide sequence" refers to a sequence, which results from reverse transcription of messenger RNA using a reverse transcriptase or any other RNA dependent DNA polymerase. Such a sequence can be
5 subsequently amplified *in vivo* or *in vitro* using a DNA dependent DNA polymerase.

As used herein the phrase "genomic polynucleotide sequence" refers to a sequence derived (isolated) from a chromosome and thus it represents a contiguous portion of a chromosome.

10 As used herein the phrase "composite polynucleotide sequence" refers to a sequence, which is at least partially complementary and at least partially genomic. A composite sequence can include some exonal sequences required to encode the polypeptide of the present invention, as well as some intronic sequences interposing therebetween. The intronic sequences can be of any source, including of other genes,
15 and typically will include conserved splicing signal sequences. Such intronic sequences may further include cis acting expression regulatory elements.

According to some embodiments of the present invention, the nucleic acid sequences encoding the polypeptides of the present invention are optimized for expression in plants. Examples of such sequence modifications include, but are not
20 limited to, an altered G/C content to more closely approach that typically found in the plant species of interest, and the removal of codons atypically found in the plant species commonly referred to as codon optimization. In one embodiment, the codon usage of the nucleic acid sequence encoding the human DNase I protein, the human DNase I protein contiguously linked at the N-terminal to an Arabidopsis ABPI endoplasmic
25 reticulum targeting signal peptide, or any other human DNase I protein described herein is optimized for *Nicotiana tabacum* or *Nicotiana benthamiana*.

The phrase "codon optimization" refers to the selection of appropriate DNA nucleotides for use within a structural gene or fragment thereof that approaches codon usage within the plant of interest. Therefore, an optimized gene or nucleic acid sequence
30 refers to a gene in which the nucleotide sequence of a native or naturally occurring gene has been modified in order to utilize statistically-preferred or statistically-favored codons within the plant. The nucleotide sequence typically is examined at the DNA

level and the coding region optimized for expression in the plant species determined using any suitable procedure, for example as described in Sardana et al. (1996, Plant Cell Reports 15:677-681). In this method, the standard deviation of codon usage, a measure of codon usage bias, may be calculated by first finding the squared proportional deviation of usage of each codon of the native gene relative to that of highly expressed plant genes, followed by a calculation of the average squared deviation. The formula used is: $SDCU = \frac{1}{N} \sum_{n=1}^N \left[\frac{X_n - Y_n}{Y_n} \right]^2$, where X_n refers to the frequency of usage of codon n in highly expressed plant genes, where Y_n to the frequency of usage of codon n in the gene of interest and N refers to the total number of codons in the gene of interest. A table of codon usage from highly expressed genes of dicotyledonous plants has been compiled using the data of Murray et al. (1989, Nuc Acids Res. 17:477-498).

One method of optimizing the nucleic acid sequence in accordance with the preferred codon usage for a particular plant cell type is based on the direct use, without performing any extra statistical calculations, of codon optimization tables such as those provided on-line at the Codon Usage Database through the NIAS (National Institute of Agrobiological Sciences) DNA bank in Japan (Hypertext Transfer Protocol/World Wide Web (dot) kazusa (dot) or (dot) jp/codon/). The Codon Usage Database contains codon usage tables for a number of different species, with each codon usage table having been statistically determined based on the data present in Genbank.

By using such codon optimization tables to determine the most preferred or most favored codons for each amino acid in a particular species (for example, rice), a naturally-occurring nucleotide sequence encoding a protein of interest can be codon optimized for that particular plant species. This is effected by replacing codons that may have a low statistical incidence in the particular species genome with corresponding codons, in regard to an amino acid, that are statistically more favored. However, one or more less-favored codons may be selected to delete existing restriction sites, to create new ones at potentially useful junctions (5' and 3' ends to add signal peptide or termination cassettes, internal sites that might be used to cut and splice segments together to produce a correct full-length sequence), or to eliminate nucleotide sequences that may negatively effect mRNA stability or expression.

The desired encoding nucleotide sequence may already, in advance of any modification, contain a number of codons that correspond to a statistically-favored codon in a particular plant species. Therefore, codon optimization of the native nucleotide sequence may comprise determining which codons, within the desired nucleotide sequence, are not statistically-favored with regards to a particular plant, and modifying these codons in accordance with a codon usage table of the particular plant to produce a codon optimized derivative. A modified nucleotide sequence may be fully or partially optimized for plant codon usage provided that the protein encoded by the modified nucleotide sequence is produced at a level higher than the protein encoded by the corresponding naturally occurring or native gene. Construction of synthetic genes by altering the codon usage is described in for example PCT Patent Application 93/07278.

According to some embodiments of the invention, the nucleic acid is operably linked to the promoter sequence.

A coding nucleic acid sequence is "operably linked" to a regulatory sequence (e.g., promoter) if the regulatory sequence is capable of exerting a regulatory effect on (e.g. effect on the expression of) the coding sequence linked thereto.

Any suitable promoter sequence can be used by the nucleic acid construct of the present invention. Preferably the promoter is a constitutive promoter, a tissue-specific, or an inducible promoter.

As used herein the phrase "plant-expressible" refers to a promoter sequence, including any additional regulatory elements added thereto or contained therein, is at least capable of inducing, conferring, activating or enhancing expression in a plant cell, tissue or organ, preferably a monocotyledonous or dicotyledonous plant cell, tissue, or organ. Such a promoter can be constitutive, i.e., capable of directing high level of gene expression in a plurality of tissues, tissue specific, i.e., capable of directing gene expression in a particular tissue or tissues, inducible, i.e., capable of directing gene expression under a stimulus, or chimeric, i.e., formed of portions of at least two different promoters.

Examples of preferred promoters useful for the methods of some embodiments of the invention are presented in Table I, II, III and IV.

Table I
**Exemplary constitutive promoters for use in the performance of some
embodiments of the invention**

Gene Source	Expression Pattern	Reference
Actin	constitutive	McElroy et al, Plant Cell, 2: 163-171, 1990
CAMV 35S	constitutive	Odell et al, Nature, 313: 810-812, 1985
CaMV 19S	constitutive	Nilsson et al., Physiol. Plant 100:456-462, 1997
GOS2	constitutive	de Pater et al, Plant J Nov;2(6):837-44, 1992
ubiquitin	constitutive	Christensen et al, Plant Mol. Biol. 18: 675-689, 1992
Rice cyclophilin	constitutive	Bucholz et al, Plant Mol Biol. 25(5):837-43, 1994
Maize H3 histone	constitutive	Lepetit et al, Mol. Gen. Genet. 231: 276-285, 1992
Actin 2	constitutive	An et al, Plant J. 10(1);107-121, 1996

5

Table II
**Exemplary seed-preferred promoters for use in the performance of some
embodiments of the invention**

Gene Source	Expression Pattern	Reference
Seed specific genes	seed	Simon, et al, Plant Mol. Biol. 5. 191, 1985; Scofield, et al., J. Biol. Chem. 262: 12202, 1987.; Baszczynski, et al., Plant Mol. Biol. 14: 633, 1990.
Brazil Nut albumin	seed	Pearson' et al., Plant Mol. Biol. 18: 235- 245, 1992.
legumin	seed	Ellis, et al.Plant Mol. Biol. 10: 203-214, 1988
Glutelin (rice)	seed	Takaiwa, et al., Mol. Gen. Genet. 208: 15-22, 1986; Takaiwa, et al., FEBS Letts. 221: 43-47, 1987
Zein	seed	Matzke et al Plant Mol Biol, 14(3)323-32 1990
napA	seed	Stalberg, et al, Planta 199: 515-519, 1996
wheat LMW and HMW, glutenin-1	endosperm	Mol Gen Genet 216:81-90, 1989; NAR 17:461-2,
Wheat SPA	seed	Albanietal, Plant Cell, 9: 171-184, 1997
wheat a, b and g gliadins	endosperm	EMB03: 1409-15, 1984
Barley ltr1 promoter	endosperm	
barley B1, C, D hordein	endosperm	Theor Appl Gen 98:1253-62, 1999; Plant J 4:343-55, 1993; Mol Gen Genet 250:750- 60, 1996

Barley DOF	endosperm	Mena et al, The Plant Journal, 116(1): 53- 62, 1998
Biz2	endosperm	EP99106056.7
Synthetic promoter	endosperm	Vicente-Carbajosa et al., Plant J. 13: 629-640, 1998
rice prolamin NRP33	endosperm	Wu et al, Plant Cell Physiology 39(8) 885- 889, 1998
rice -globulin Glb-1	endosperm	Wu et al, Plant Cell Physiology 39(8) 885-889, 1998
rice OSH1	embryo	Sato et al, Proc. Nati. Acad. Sci. USA, 93: 8117-8122
rice alpha-globulin REB/OHP-1	endosperm	Nakase et al. Plant Mol. Biol. 33: 513-S22, 1997
rice ADP-glucose PP	endosperm	Trans Res 6:157-68, 1997
maize ESR gene family	endosperm	Plant J 12:235-46, 1997
sorghum gamma- kafirin	endosperm	PMB 32:1029-35, 1996
KNOX	emryo	Postma-Haarsma ef al, Plant Mol. Biol. 39:257-71, 1999
rice oleosin	Embryo and aleuton	Wu et at, J. Biochem., 123:386, 1998
sunflower oleosin	Seed (embryo and dry seed)	Cummins, etal., Plant Mol. Biol. 19: 873- 876, 1992

Table III
Exemplary flower-specific promoters for use in the performance of the invention

5

<i>Gene Source</i>	<i>Expression Pattern</i>	<i>Reference</i>
AtPRP4	flowers	www.salus. medium.edu/m mg/tierney/html
chalcone synthase (chsA)	flowers	Van der Meer, et al., Plant Mol. Biol. 15, 95-109, 1990.
LAT52	anther	Twel et al Mol. Gen Genet. 217:240-245 (1989)
apetala- 3	flowers	

Table IV
Alternative rice promoters for use in the performance of the invention

10

<i>PRO #</i>	<i>gene</i>	<i>expression</i>
PR00001	Metallothionein Mte	transfer layer of embryo + calli
PR00005	putative beta-amylase	transfer layer of embryo
PR00009	Putative cellulose synthase	Weak in roots
PR00012	lipase (putative)	
PR00014	Transferase (putative)	
PR00016	peptidyl prolyl cis-trans isomerase (putative)	
PR00019	unknown	
PR00020	prp protein (putative)	
PR00029	noduline (putative)	
PR00058	Proteinase inhibitor Rgpi9	seed

PR00061	beta expansine EXPB9	Weak in young flowers
PR00063	Structural protein	young tissues+calli+embryo
PR00069	xylosidase (putative)	
PR00075	Prolamine 10Kda	strong in endosperm
PR00076	allergen RA2	strong in endosperm
PR00077	prolamine RP7	strong in endosperm
PR00078	CBP80	
PR00079	starch branching enzyme I	
PR00080	Metallothioneine-like ML2	transfer layer of embryo + calli
PR00081	putative caffeoyl- CoA 3-0 methyltransferase	shoot
PR00087	prolamine RM9	strong in endosperm
PR00090	prolamine RP6	strong in endosperm
PR00091	prolamine RP5	strong in endosperm
PR00092	allergen RA5	
PR00095	putative methionine aminopeptidase	embryo
PR00098	ras-related GTP binding protein	
PR00104	beta expansine EXPB 1	
PR00105	Glycine rich protein	
PR00108	metallothionein like protein (putative)	
PR00110	RCc3 strong root	
PR00111	uclacyanin 3-like protein	weak discrimination center / shoot meristem
PR00116	26S proteasome regulatory particle non-ATPase subunit 11	very weak meristem specific
PR00117	putative 40S ribosomal protein	weak in endosperm
PR00122	chlorophyll a/lb-binding protein precursor (Cab27)	very weak in shoot
PR00123	putative protochlorophyllide reductase	Strong leaves
PR00126	metallothionein RiCMT	strong discrimination center shoot meristem
PR00129	GOS2	Strong constitutive
PR00131	GOS9	
PR00133	chitinase Cht-3	very weak meristem specific
PR00135	alpha- globulin	Strong in endosperm
PR00136	alanine aminotransferase	Weak in endosperm
PR00138	Cyclin A2	
PR00139	Cyclin D2	
PR00140	Cyclin D3	
PR00141	Cyclophyllin 2	Shoot and seed
PR00146	sucrose synthase SSI (barley)	medium constitutive
PR00147	trypsin inhibitor ITR1 (barley)	weak in endosperm
PR00149	ubiquitin 2 with intron	strong constitutive
PR00151	WSI18	Embryo and stress
PR00156	HVA22 homologue (putative)	
PR00157	EL2	
PR00169	aquaporine	medium constitutive in young plants
PR00170	High mobility group protein	Strong constitutive
PR00171	reversibly glycosylated protein RGP1	weak constitutive
PR00173	cytosolic MDH	shoot
PR00175	RAB21	Embryo and stress

PRO0176	CDPK7	
PRO0177	Cdc2-1	very weak in meristem
PRO0197	sucrose synthase 3	
PRO0198	OsVPI	
PRO0200	OSH1	very weak in young plant meristem
PRO0208	putative chlorophyllase	
PRO0210	OsNRT1	
PRO021 1	EXP3	
PRO0216	phosphate transporter OjPT1	
PRO0218	oleosin 18kd	aleurone + embryo
PRO0219	ubiquitin 2 without intron	
PRO0220	RFL	
PRO0221	maize UBI delta intron	not detected
PRO0223	glutelin-1	
PRO0224	fragment of prolamin RP6 promoter	
PRO0225	4xABRE	
PRO0226	glutelin OSGLUA3	
PRO0227	BLZ-2_short (barley)	
PRO0228	BLZ-2_long (barley)	

The nucleic acid construct of some embodiments of the invention can further include an appropriate selectable marker and/or an origin of replication. According to some embodiments of the invention, the nucleic acid construct utilized is a shuttle vector, which can propagate both in E. coli (wherein the construct comprises an appropriate selectable marker and origin of replication) and be compatible with propagation in cells. The construct according to the present invention can be, for example, a plasmid, a bacmid, a phagemid, a cosmid, a phage, a virus or an artificial chromosome.

The nucleic acid construct of some embodiments of the invention can be utilized to stably or transiently transform plant cells. In stable transformation, the nucleic acid is integrated into the plant genome and as such it represents a stable and inherited trait. In transient transformation, the exogenous polynucleotide is expressed by the cell transformed but it is not integrated into the genome and as such it represents a transient trait.

Thus, according to some aspects of the present invention, there is provided an isolated cell comprising the nucleic acid construct of the invention.

As used herein, the term "isolated cell" refers to a cell at least partially separated from the natural environment e.g., from a plant. In some embodiments, the isolated cell

is a plant cell of a whole plant. In some embodiments, the isolated cell is a plant cell, for example, a plant cell in culture.

The term "plant" as used herein encompasses whole plants, ancestors and progeny of the plants and plant parts, including seeds, shoots, stems, roots (including tubers), and plant cells, tissues and organs. The plant may be in any form including suspension cultures, embryos, meristematic regions, callus tissue, leaves, gametophytes, sporophytes, pollen, and microspores. Plants that are particularly useful in the methods of the invention include all plants which belong to the superfamily Viridiplantae, in particular monocotyledonous and dicotyledonous plants including a fodder or forage legume, ornamental plant, food crop, tree, or shrub selected from the list comprising

Acacia spp., Acer spp., Actinidia spp., Aesculus spp., Agathis australis, Albizia amara, Alsophila tricolor, Andropogon spp., Arachis spp, Areca catechu, Astelia fragrans, Astragalus cicer, Baikiaea plurijuga, Betula spp., Brassica spp., Bruguiera gymnorrhiza, Burkea africana, Butea frondosa, Cadaba farinosa, Calliandra spp, Camellia sinensis, Canna indica, Capsicum spp., Cassia spp., Centroema pubescens, Chacoomeles spp., Cinnamomum cassia, Coffea arabica, Colophospermum mopane, Coronillia varia, Cotoneaster serotina, Crataegus spp., Cucumis spp., Cupressus spp., Cyathea dealbata, Cydonia oblonga, Cryptomeria japonica, Cymbopogon spp., Cynthea dealbata, Cydonia oblonga, Dalbergia monetaria, Davallia divaricata, Desmodium spp., Dicksonia squarosa, Dibeteropogon amplexans, Dioclea spp, Dolichos spp., Dorycnium rectum, Echinochloa pyramidalis, Ehraffia spp., Eleusine coracana, Eragrostis spp., Erythrina spp., Eucalyptus spp., Euclea schimperi, Eulalia villosa, Pagopyrum spp., Feijoa sellowiana, Fragaria spp., Flemingia spp, Freycinetia banksii, Geranium thunbergii, GinAgo biloba, Glycine javanica, Gliricidia spp, Gossypium hirsutum, Grevillea spp., Guibourtia coleosperma, Hedysarum spp., Hemaphysalis altissima, Heteropogon contortus, Hordeum vulgare, Hyparrhenia rufa, Hypericum erectum, Hypochaeris glabra, Indigo tinctoria, Iris spp., Leptarrhena pyrolifolia, Lespedeza spp., Lettuce spp., Leucaena leucocephala, Loudetia simplex, Lotonotus bainesii, Lotus spp., Macrotyloma axillare, Malus spp., Manihot esculenta, Medicago sativa, Metasequoia glyptostroboides, Musa sapientum, Nicotiana spp., Onobrychis spp., Ornithopus spp., Oryza spp., Peltophorum africanum, Pennisetum spp., Persea gratissima, Petunia spp., Phaseolus spp., Phoenix canariensis, Phormium cookianum, Photinia spp., Picea glauca, Pinus spp.,

Pisum sativum, Podocarpus totara, Pogonarthria fleckii, Pogonaffhria squarrosa, Populus spp., Prosopis cineraria, Pseudotsuga menziesii, Pterolobium stellatum, Pyrus communis, Quercus spp., Rhamphiolepis umbellata, Rhopalostylis sapida, Rhus natalensis, Ribes grossularia, Ribes spp., Robinia pseudoacacia, Rosa spp., Rubus spp.,
5 Salix spp., Schyzachyrium sanguineum, Sciadopitys vefficillata, Sequoia sempervirens, Sequoiadendron giganteum, Sorghum bicolor, Spinacia spp., Sporobolus fimbriatus, Stiburus alopecuroides, Stylosanthos humilis, Tadehagi spp, Taxodium distichum, Themeda triandra, Trifolium spp., Triticum spp., Tsuga heterophylla, Vaccinium spp., Vicia spp., Vitis vinifera, Watsonia pyramidata, Zantedeschia aethiopica, Zea mays,
10 amaranth, artichoke, asparagus, broccoli, Brussels sprouts, cabbage, canola, carrot, cauliflower, celery, collard greens, flax, kale, lentil, oilseed rape, okra, onion, potato, rice, soybean, straw, sugar beet, sugar cane, sunflower, tomato, squash tea, maize, wheat, barely, rye, oat, peanut, pea, lentil and alfalfa, cotton, rapeseed, canola, pepper, sunflower, tobacco, eggplant, eucalyptus, a tree, an ornamental plant, a perennial grass
15 and a forage crop. Alternatively algae and other non-Viridiplantae can be used for the methods of the present invention.

According to some embodiments of the invention, the plant or plant cell is a duckweed plant, cell or nodule. Duckweed (members of the monocotyledonous family Lemnaceae, or Lemna) plant or duckweed nodule cultures can be efficiently transformed
20 with an expression cassette containing a nucleotide sequence of interest by any one of a number of methods including Agrobacterium-mediated gene transfer, ballistic bombardment, or electroporation. Methods for molecular engineering of duckweed cells and detailed description of duckweed expression systems and useful for commercial production of valuable polypeptides are known in the art (see, for example, US Patent
25 Nos. 6,040,498 and 6,815,184 to Stomp, et al, and 8,022,270 to Dickey et al).

According to some embodiments of the invention, the plant or plant cell used by the method of the invention is a crop plant or cell of a crop plant such as rice, maize, wheat, barley, peanut, potato, sesame, olive tree, palm oil, banana, soybean, sunflower, canola, sugarcane, alfalfa, millet, leguminosae (bean, pea), flax, lupinus, rapeseed,
30 tobacco, poplar and cotton.

According to further embodiments the plant cells includes tobacco cells, *Agrobacterium rhizogenes* transformed root cell, celery cell, ginger cell, horseradish

cell and carrot cells. In one embodiment the tobacco cells are from a tobacco cell line, such as, but not limited to *Nicotiana tabacum* L. cv Bright Yellow (BY-2) cells. The plant cells may be grown according to any type of suitable culturing method, including but not limited to, culture on a solid surface (such as a plastic culturing vessel or plate for example) or in suspension. It will be noted that some cells, such as the BY-2 and carrot cells can be cultured and grown in suspension. Suitable devices and methods for culturing plant cells in suspension are known in the art, for example, as described in International Patent Application PCT IL2008/000614. In yet another embodiment the cells are cells of whole tobacco plants or plant tissues, including, but not limited to *Nicotiana benthamiana*.

There are various methods of introducing foreign genes into both monocotyledonous and dicotyledonous plants (Potrykus, I., Annu. Rev. Plant. Physiol., Plant. Mol. Biol. (1991) 42:205-225; Shimamoto et al., Nature (1989) 338:274-276).

The principle methods of causing stable integration of exogenous DNA into plant genomic DNA include two main approaches:

(i) Agrobacterium-mediated gene transfer: Klee et al. (1987) Annu. Rev. Plant Physiol. 38:467-486; Klee and Rogers in Cell Culture and Somatic Cell Genetics of Plants, Vol. 6, Molecular Biology of Plant Nuclear Genes, eds. Schell, J., and Vasil, L. K., Academic Publishers, San Diego, Calif. (1989) p. 2-25; Gatenby, in Plant Biotechnology, eds. Kung, S. and Arntzen, C. J., Butterworth Publishers, Boston, Mass. (1989) p. 93-112.

(ii) Direct DNA uptake: Paszkowski et al., in Cell Culture and Somatic Cell Genetics of Plants, Vol. 6, Molecular Biology of Plant Nuclear Genes eds. Schell, J., and Vasil, L. K., Academic Publishers, San Diego, Calif. (1989) p. 52-68; including methods for direct uptake of DNA into protoplasts, Toriyama, K. et al. (1988) Bio/Technology 6:1072-1074. DNA uptake induced by brief electric shock of plant cells: Zhang et al. Plant Cell Rep. (1988) 7:379-384. Fromm et al. Nature (1986) 319:791-793. DNA injection into plant cells or tissues by particle bombardment, Klein et al. Bio/Technology (1988) 6:559-563; McCabe et al. Bio/Technology (1988) 6:923-926; Sanford, Physiol. Plant. (1990) 79:206-209; by the use of micropipette systems: Neuhaus et al., Theor. Appl. Genet. (1987) 75:30-36; Neuhaus and Spangenberg, Physiol. Plant. (1990) 79:213-217; glass fibers or silicon carbide whisker transformation

of cell cultures, embryos or callus tissue, U.S. Pat. No. 5,464,765 or by the direct incubation of DNA with germinating pollen, DeWet et al. in *Experimental Manipulation of Ovule Tissue*, eds. Chapman, G. P. and Mantell, S. H. and Daniels, W. Longman, London, (1985) p. 197-209; and Ohta, *Proc. Natl. Acad. Sci. USA* (1986) 83:715-
5 719.

The *Agrobacterium* system includes the use of plasmid vectors that contain defined DNA segments that integrate into the plant genomic DNA. Methods of inoculation of the plant tissue vary depending upon the plant species and the *Agrobacterium* delivery system. A widely used approach is the leaf disc procedure
10 which can be performed with any tissue explant that provides a good source for initiation of whole plant differentiation. See, e.g., Horsch et al. in *Plant Molecular Biology Manual A5*, Kluwer Academic Publishers, Dordrecht (1988) p. 1-9. A supplementary approach employs the *Agrobacterium* delivery system in combination with vacuum infiltration. The *Agrobacterium* system is especially viable in the creation
15 of transgenic dicotyledonous plants.

There are various methods of direct DNA transfer into plant cells. In electroporation, the protoplasts are briefly exposed to a strong electric field. In microinjection, the DNA is mechanically injected directly into the cells using very small micropipettes. In microparticle bombardment, the DNA is adsorbed on microprojectiles
20 such as magnesium sulfate crystals or tungsten particles, and the microprojectiles are physically accelerated into cells or plant tissues.

Following stable transformation plant propagation is exercised. The most common method of plant propagation is by seed. Regeneration by seed propagation, however, has the deficiency that due to heterozygosity there is a lack of uniformity in
25 the crop, since seeds are produced by plants according to the genetic variances governed by Mendelian rules. Basically, each seed is genetically different and each will grow with its own specific traits. Therefore, it is preferred that the transformed plant be produced such that the regenerated plant has the identical traits and characteristics of the parent transgenic plant. Therefore, it is preferred that the transformed plant be
30 regenerated by micropropagation which provides a rapid, consistent reproduction of the transformed plants.

Micropropagation is a process of growing new generation plants from a single piece of tissue that has been excised from a selected parent plant or cultivar. This process permits the mass reproduction of plants having the preferred tissue expressing the fusion protein. The new generation plants which are produced are genetically identical to, and have all of the characteristics of, the original plant. Micropropagation allows mass production of quality plant material in a short period of time and offers a rapid multiplication of selected cultivars in the preservation of the characteristics of the original transgenic or transformed plant. The advantages of cloning plants are the speed of plant multiplication and the quality and uniformity of plants produced.

Micropropagation is a multi-stage procedure that requires alteration of culture medium or growth conditions between stages. Thus, the micropropagation process involves four basic stages: Stage one, initial tissue culturing; stage two, tissue culture multiplication; stage three, differentiation and plant formation; and stage four, greenhouse culturing and hardening. During stage one, initial tissue culturing, the tissue culture is established and certified contaminant-free. During stage two, the initial tissue culture is multiplied until a sufficient number of tissue samples are produced to meet production goals. During stage three, the tissue samples grown in stage two are divided and grown into individual plantlets. At stage four, the transformed plantlets are transferred to a greenhouse for hardening where the plants' tolerance to light is gradually increased so that it can be grown in the natural environment.

According to some embodiments of the invention, the transgenic plants are generated by transient transformation of leaf cells, meristematic cells or the whole plant.

Transient transformation can be effected by any of the direct DNA transfer methods described above or by viral infection using modified plant viruses.

Viruses that have been shown to be useful for the transformation of plant hosts include CaMV, Tobacco mosaic virus (TMV), brome mosaic virus (BMV) and Bean Common Mosaic Virus (BV or BCMV). Transformation of plants using plant viruses is described in U.S. Pat. No. 4,855,237 (bean golden mosaic virus; BGV), EP-A 67,553 (TMV), Japanese Published Application No. 63-14693 (TMV), EPA 194,809 (BV), EPA 278,667 (BV); and Gluzman, Y. et al., Communications in Molecular Biology: Viral Vectors, Cold Spring Harbor Laboratory, New York, pp. 172-189 (1988).

Pseudovirus particles for use in expressing foreign DNA in many hosts, including plants are described in WO 87/06261.

According to some embodiments of the invention, the virus used for transient transformations is avirulent and thus is incapable of causing severe symptoms such as reduced growth rate, mosaic, ring spots, leaf roll, yellowing, streaking, pox formation, tumor formation and pitting. A suitable avirulent virus may be a naturally occurring avirulent virus or an artificially attenuated virus. Virus attenuation may be effected by using methods well known in the art including, but not limited to, sub-lethal heating, chemical treatment or by directed mutagenesis techniques such as described, for example, by Kurihara and Watanabe (*Molecular Plant Pathology* 4:259-269, 2003), Galon et al. (1992), Atreya et al. (1992) and Huet et al. (1994).

Suitable virus strains can be obtained from available sources such as, for example, the American Type Culture Collection (ATCC) or by isolation from infected plants. Isolation of viruses from infected plant tissues can be effected by techniques well known in the art such as described, for example by Foster and Tatlor, Eds. "Plant Virology Protocols: From Virus Isolation to Transgenic Resistance (Methods in Molecular Biology (Humana Pr), Vol 81)", Humana Press, 1998. Briefly, tissues of an infected plant believed to contain a high concentration of a suitable virus, preferably young leaves and flower petals, are ground in a buffer solution (e.g., phosphate buffer solution) to produce a virus infected sap which can be used in subsequent inoculations.

Construction of plant RNA viruses for the introduction and expression of non-viral nucleic acid sequences in plants is demonstrated by the above references as well as by Dawson, W. O. et al., *Virology* (1989) 172:285-292; Takamatsu et al. *EMBO J.* (1987) 6:307-311; French et al. *Science* (1986) 231:1294-1297; Takamatsu et al. *FEBS Letters* (1990) 269:73-76; and U.S. Pat. No. 5,316,931.

When the virus is a DNA virus, suitable modifications can be made to the virus itself. Alternatively, the virus can first be cloned into a bacterial plasmid for ease of constructing the desired viral vector with the foreign DNA. The virus can then be excised from the plasmid. If the virus is a DNA virus, a bacterial origin of replication can be attached to the viral DNA, which is then replicated by the bacteria. Transcription and translation of this DNA will produce the coat protein which will encapsidate the viral DNA. If the virus is an RNA virus, the virus is generally cloned as a cDNA and

inserted into a plasmid. The plasmid is then used to make all of the constructions. The RNA virus is then produced by transcribing the viral sequence of the plasmid and translation of the viral genes to produce the coat protein(s) which encapsidate the viral RNA.

5 In one embodiment, a plant viral polynucleotide is provided in which the native coat protein coding sequence has been deleted from a viral polynucleotide, a non-native plant viral coat protein coding sequence and a non-native promoter, preferably the subgenomic promoter of the non-native coat protein coding sequence, capable of expression in the plant host, packaging of the recombinant plant viral polynucleotide,
10 and ensuring a systemic infection of the host by the recombinant plant viral polynucleotide, has been inserted. Alternatively, the coat protein gene may be inactivated by insertion of the non-native polynucleotide sequence within it, such that a protein is produced. The recombinant plant viral polynucleotide may contain one or more additional non-native subgenomic promoters. Each non-native subgenomic
15 promoter is capable of transcribing or expressing adjacent genes or polynucleotide sequences in the plant host and incapable of recombination with each other and with native subgenomic promoters. Non-native (foreign) polynucleotide sequences may be inserted adjacent the native plant viral subgenomic promoter or the native and a non-native plant viral subgenomic promoters if more than one polynucleotide sequence is
20 included. The non-native polynucleotide sequences are transcribed or expressed in the host plant under control of the subgenomic promoter to produce the desired products.

In a second embodiment, a recombinant plant viral polynucleotide is provided as in the first embodiment except that the native coat protein coding sequence is placed adjacent one of the non-native coat protein subgenomic promoters instead of a non-
25 native coat protein coding sequence.

In a third embodiment, a recombinant plant viral polynucleotide is provided in which the native coat protein gene is adjacent its subgenomic promoter and one or more non-native subgenomic promoters have been inserted into the viral polynucleotide. The inserted non-native subgenomic promoters are capable of transcribing or expressing
30 adjacent genes in a plant host and are incapable of recombination with each other and with native subgenomic promoters. Non-native polynucleotide sequences may be inserted adjacent the non-native subgenomic plant viral promoters such that the

sequences are transcribed or expressed in the host plant under control of the subgenomic promoters to produce the desired product.

In a fourth embodiment, a recombinant plant viral polynucleotide is provided as in the third embodiment except that the native coat protein coding sequence is replaced
5 by a non-native coat protein coding sequence.

The viral vectors are encapsidated by the coat proteins encoded by the recombinant plant viral polynucleotide to produce a recombinant plant virus. The recombinant plant viral polynucleotide or recombinant plant virus is used to infect appropriate host plants. The recombinant plant viral polynucleotide is capable of
10 replication in the host, systemic spread in the host, and transcription or expression of foreign gene(s) (exogenous polynucleotide) in the host to produce the desired protein.

Techniques for inoculation of viruses to plants may be found in Foster and Taylor, eds. "Plant Virology Protocols: From Virus Isolation to Transgenic Resistance (Methods in Molecular Biology (Humana Pr), Vol 81)", Humana Press, 1998;
15 Maramorosh and Koprowski, eds. "Methods in Virology" 7 vols, Academic Press, New York 1967-1984; Hill, S.A. "Methods in Plant Virology", Blackwell, Oxford, 1984; Walkey, D.G.A. "Applied Plant Virology", Wiley, New York, 1985; and Kado and Agrawa, eds. "Principles and Techniques in Plant Virology", Van Nostrand-Reinhold, New York.

20 In addition to the above, the polynucleotide of the present invention can also be introduced into a chloroplast genome thereby enabling chloroplast expression.

A technique for introducing exogenous nucleic acid sequences to the genome of the chloroplasts is known. This technique involves the following procedures. First, plant cells are chemically treated so as to reduce the number of chloroplasts per cell to
25 about one. Then, the exogenous polynucleotide is introduced via particle bombardment into the cells with the aim of introducing at least one exogenous polynucleotide molecule into the chloroplasts. The exogenous polynucleotides selected such that it is integratable into the chloroplast's genome via homologous recombination which is readily effected by enzymes inherent to the chloroplast. To this end, the nucleic acid
30 sequence includes, in addition to a gene of interest, at least one polynucleotide stretch which is derived from the chloroplast's genome. In addition, the exogenous polynucleotide includes a selectable marker, which serves by sequential selection

procedures to ascertain that all or substantially all of the copies of the chloroplast genomes following such selection will include the exogenous polynucleotide. Further details relating to this technique are found in U.S. Pat. Nos. 4,945,050; and 5,693,507 which are incorporated herein by reference. A polypeptide can thus be produced by the protein expression system of the chloroplast and become integrated into the chloroplast's inner membrane.

According to some embodiments of the invention, the method further comprises growing the plant cell expressing the nucleic acid. The plant cells can be any plant cells desired. The plant cells can be cultured cells, cells in cultured tissue or cultured organs, or cells in a plant. In some embodiments, the plant cells are cultured cells, or cells in cultured tissue or cultured organs. In yet further embodiments, the plant cells are any type of plant that is used in gene transference. The plant cell can be grown as part of a whole plant, or, alternatively, in plant cell culture.

According to some aspects of the invention, the plant cells are grown in a plant cell suspension culture. As used herein, the term "suspension culture" refers to the growth of cells separate from the organism. Suspension culture can be facilitated via use of a liquid medium (a "suspension medium"). Suspension culture can refer to the growth of cells in liquid nutrient media. Methods and devices suitable for growing plant cells of the invention in plant cell suspension culture are described in detail in, for example, PCT WO2008/135991, US Patent No. 6,391,683, US Patent Application No. 10/784,295; International Patent Publications PCT Nos. WO2004/091475, WO2005/080544 and WO 2006/040761, all of which are hereby incorporated by reference as if fully set forth herein.

Thus, the invention encompasses plants or plant cultures expressing the nucleic acid sequences, so as to produce the recombinant human DNase I protein of the invention. Once expressed within the plant cell or the entire plant, the level of the human DNase I protein encoded by the nucleic acid sequence can be determined by methods well known in the art such as, activity assays, Western blots using antibodies capable of specifically binding the human DNase I protein, Enzyme-Linked Immuno Sorbent Assay (ELISA), radio-immuno-assays (RIA), immunohistochemistry, immunocytochemistry, immunofluorescence and the like.

Methods of determining the level in the plant of the RNA transcribed from the nucleic acid sequence are well known in the art and include, for example, Northern blot analysis, reverse transcription polymerase chain reaction (RT-PCR) analysis (including quantitative, semi-quantitative or real-time RT-PCR) and RNA-*in situ* hybridization.

5 According to some embodiments of the invention, the expressed recombinant human DNase I protein is glycosylated in the plant cell, resulting in a recombinant human DNase I protein having high mannose glycosylation (e.g. exposed mannose sugar residues), and plant specific glycan residues. Thus, according to some embodiments of the invention, the cells expressing the expression vector of the invention produce a
10 human DNase I protein having at least one, optionally at least two, optionally at least three or optionally at least four or more exposed mannose residues. In other embodiments the cells expressing the expression vector of the invention produce a human DNase I protein having at least one, optionally at least two, optionally at least three or optionally at least four or more core xylose residues. In yet other embodiments
15 the cells expressing the expression vector of the invention produce a human DNase I protein having at least one, optionally at least two, optionally at least three or optionally at least four or more core α -(1,3) fucose residues. In one embodiment the cells expressing the expression vector of the invention produce a human DNase I protein having at least one exposed mannose residue, at least one core xylose residue and at
20 least one α -(1,3) fucose residue. In yet further embodiments, the cells expressing the expression vector of the invention produce a human DNase I protein having at least one, at least two, at least 3 or more terminal N-acetyl glucosamine substitutions on the outer mannose sugars.

Glycan analysis of human DNase I protein expressed in plant cells indicated that
25 the prh DNase I lacks sialic acid residues. Thus, in yet other embodiments, the cells expressing the expression vector of the invention produce a recombinant human DNase I protein devoid of sialic acid residues.

The human DNase I protein produced by cells expressing the expression vector of the invention was shown to have DNase I catalytic activity similar or superior to that
30 of a native human or mammalian-cell produced DNase I enzyme.

As shown in Example 3 herein, when enzyme kinetics of the plant-expressed recombinant human DNase I protein were measured (see Table VI), greater substrate

affinity (K_M) and higher reaction velocity (V_{max}) were observed for the plant-expressed recombinant human DNase I protein, as compared to those of mammalian cell-expressed recombinant human DNase I (e.g. Pulmozyme ®). Thus, according to some embodiments the plant expressed recombinant human DNase I is biologically active. In some embodiments, the biological activity is endonuclease catalytic activity. In yet other embodiments, the biological activity of the plant-expressed recombinant human DNase I protein is characterized by greater substrate affinity (K_M) and higher reaction velocity (V_{max}), as compared to those of mammalian cell-expressed recombinant human DNase I (e.g. Pulmozyme ®). Methods for measuring biological activity of DNase I are well known in the art, and include, inter alia, catalytic activity (e.g. methyl green assay, DNaseAlert™-based assay, fluorescence-based assay, as described herewith), immunoreactivity with anti-DNase I antibody, Kunitz-based hyperchromicity assay, assays measuring proton release upon DNA hydrolysis, which can be monitored by using chromophoric H⁺ ion indicators, and the like. In still other embodiments, the biological activity of the plant-expressed recombinant human DNase I protein is characterized by specific activity (units DNase I catalytic activity per mg enzyme protein) greater than that of mammalian cell-expressed recombinant human DNase I (e.g. Pulmozyme ®). In some embodiments, the specific activity of plant-expressed recombinant human DNase I protein is about 1.1, about 1.2, about 1.25, about 1.5, about 1.75, about 2.0, about 2.25, about 2.5, about 2.75, about 3.0, about 3.25, about 3.5, about 3.75, about 4.0, about 4.5 to 5.0 or more fold that of the specific activity (e.g. U/mg) of mammalian cell-expressed recombinant human DNase I (e.g. Pulmozyme ®). In some embodiments, the specific activity of plant-expressed recombinant human DNase I protein is about 3.0 to 3.5 fold that of the specific activity (e.g. U/mg) of mammalian cell-expressed recombinant human DNase I (e.g. Pulmozyme ®).

As shown in Example 4 herein, when enzyme activity of DNase I protein was measured (see Table VIII) in the presence of increasing concentrations of actin, greater resistance of the plant-expressed recombinant human DNase I to inhibition of catalytic activity by actin was observed, compared to that of mammalian cell-expressed recombinant human DNase I (e.g. Pulmozyme ®). Thus, according to some embodiments the plant expressed recombinant human DNase I has greater resistance to actin inhibition of DNase I catalytic activity when compared to that of mammalian cell-

expressed recombinant human DNase I (e.g. Pulmozyme ®). In some embodiments, the inhibition of DNase I catalytic activity is expressed as half-maximal inhibitory concentration of actin (IC_{50} , $\mu\text{g actin/ml}$), using, for example, the methyl green DNase I assay. Thus, in some embodiments, the IC_{50} actin concentration for inhibition of the plant expressed recombinant human DNase I is at least 1.25, about 1.5, about 1.75, about 2.0, about 2.25, about 2.5, about 2.75, about 3.0, about 3.25, about 3.5, about 3.75, about 4.0, about 4.5 to 5.0 or more, or a range of about 1.5 to 2.5 fold greater than that of mammalian cell-expressed recombinant human DNase I (e.g. Pulmozyme ®). In some embodiments, the IC_{50} actin concentration for inhibition of the plant expressed recombinant human DNase I activity is about 2.0 to 2.2 fold that of mammalian cell-expressed recombinant human DNase I (e.g. Pulmozyme ®).

As shown in Example 5, plant expressed recombinant human DNase I effectively reduces the rheological properties of sputum. When measured in an *in-vitro* assay, incubation of the plant expressed recombinant human DNase I with samples of CF sputum results in reduction of the viscous modulus (as expressed by the loss modulus, G'') and reduction of the elasticity (as expressed by the storage modulus, G') of the sputum sample (see Figures 12A-12D, 13A-13D, 14A-14B, 16 and 17). When compared to the reduction of rheological parameters of sputum samples incubated with mammalian cell-expressed recombinant human DNase I (e.g. Pulmozyme ®), the plant expressed recombinant human DNase I displayed greater efficacy in reducing viscosity and elasticity than that of mammalian cell-expressed recombinant human DNase I (e.g. Pulmozyme ®) (Figures 12 and 13). Thus, according to some embodiments of the invention, the plant expressed recombinant human DNase I reduces viscous modulus and/or elastic modulus of sputum. In other embodiments, reduction in viscous and elastic modulus is expressed as G'' and G' , respectively. In yet another embodiment, reduction of viscous and/or elastic modulus of sputum by the plant expressed recombinant human DNase I is greater than that of mammalian cell-expressed recombinant human DNase I (e.g. Pulmozyme ®) measured with in the same assay technique. Thus, in some embodiments, the plant expressed human recombinant DNase I of the invention is biologically active, having catalytic activity, enzyme kinetics and specific activity comparable or superior to that of mammalian cell-expressed recombinant human DNase I, and effective in reducing rheological properties of CF

sputum. In other embodiments, the rheological properties of sputum are assayed using stress sweep measurements, and can be characterized by the cross-over points of elastic and viscous stress values.

Thus, the human DNase I protein expressed in plant cells according to the invention can be used to produce an inhalable dry powder formulation for treatment or prevention of any condition or disease by pulmonary administration. According to some aspects of the invention, the dry powder formulation can be used for treatment or prevention of mucus-associated conditions in a subject in need thereof. In some embodiments, the mucus associated conditions comprise respiratory or pulmonary disease or conditions.

In some embodiments of the invention, the dry powder formulation comprising DNase I can be administered for reducing extracellular DNA in a secretion, fluid or tissue of a subject in need thereof. Secretions, tissues and fluids accessible to the dry powder formulations of the invention include pulmonary secretions, fluids and tissues such as mucus and other extracellular bronchopulmonary fluids, and secretions, fluids and tissues accessible via the circulatory system, such as blood, intestinal mucosal secretions and the like.

Thus, according to some embodiments of the invention, there is provided a method for preventing or treating a pulmonary disease or condition associated with excess DNA in a pulmonary secretion in a subject in need thereof, the method comprising administering, via pulmonary administration, an effective amount of the dry powder inhalable formulation of the invention. Suitable formulations and dosage regimens are provided herewith in detail.

Respiratory conditions or diseases which can be treated by administration of the dry powder formulation of the invention include conditions associated with accumulation of mucus or other DNA containing secretions or fluids, for example, in the airways. Such conditions include, but are not limited to acute or chronic bronchopulmonary disease, atelectasis due to tracheal or bronchial impaction and complications of tracheostomy chronic bronchitis, asthmatic bronchitis, cystic fibrosis, pneumonia, allergic diseases such as allergic asthma, non-allergic asthma, systemic lupus erythematosus, Sjogren's syndrome, bronchiectasis, emphysema, acute and chronic sinusitis, and even the common cold.

Non-respiratory conditions that can be treated by the dry powder formulations of the invention include, but are not limited to, male infertility, metastatic cancer, viral, bacterial, fungal and protozoan infections and sepsis, atherosclerosis, diabetes, delayed type hypersensitivity and uterine disorders.

5 In further embodiments, biological activity of the plant expressed recombinant human DNase I of the invention is enhanced by the presence of an additional pharmacological agent, for example, an agent which reduces actin inhibition of DNase activity, such as one or more inorganic salt selected from potassium, magnesium, calcium, zinc, lithium, manganese, cadmium, nickel, cobalt, ammonium, polyamine and
10 macrocyclic polyammonium salts. Agents suitable for combination with the plant expressed human recombinant DNase I of the invention, for therapeutic applications such as treatment of pulmonary conditions (e.g. CF) are described in detail in US 7,432,308 to Demeester et al., which is incorporated herein by reference in its entirety.

In some embodiments, combination of the plant expressed recombinant human
15 DNase I with an additional pharmaceutical agent results in improvement, and optionally synergic improvement in reduction of rheological properties (e.g. viscous modulus and/or elastic modulus) of sputum. In some embodiments, the additional agent is magnesium chloride or magnesium sulfate.

In some embodiments of the invention, the dry powder formulation comprising
20 DNase I is combined with, or administered along with an additional pharmaceutical agent, the additional pharmaceutical agent including, but not limited to one or more other pharmacologic agents used to treat the conditions listed above, such as antibiotics, bronchodilators, anti-inflammatory agents, mucolytics (e.g. n-acetyl-cysteine), actin binding or actin severing proteins [e.g., gelsolin; Matsudaira et al., Cell 54:139-140
25 (1988); Stossel, et al., PCT Patent Publication No. WO 94/22465], protease inhibitors, or gene therapy product [e.g., comprising the cystic fibrosis transmembrane conductance regulator (CFTR) gene, Riordan, et al., Science 245:1066-1073 (1989)]. Additional pharmaceutical agents can be administered prior to, along with, subsequent to or in any other temporal combination with the formulation of the invention. Regimen for
30 combination of the formulation of the invention with additional agents can be formulated according to parameters such as specific conditions or diseases, health status of the subject, methods and dose of administration, and the like. Determination of such

combination regimen can be done, for example, by professionals such as attending physicians, hospital staff, and also according to predetermined protocols.

Herein the term "active ingredient" refers to at least the recombinant human DNase I protein (and optionally at least one of the above pharmaceutical agents) accountable for the biological effect.

Hereinafter, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. An adjuvant is included under these phrases.

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

Techniques for formulation and administration of drugs may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

As used herein, the term "subject in need thereof" refers to a subject diagnosed with or exhibiting one or more conditions associated with a disease or condition treatable by administration of DNase I, a subject who has been diagnosed with or exhibited one or more conditions treatable by administration of DNase I in the past, or a subject who has been deemed at risk of developing one or more conditions associated with a disease or condition treatable by administration of DNase I in the future due to hereditary or environmental factors. In certain embodiments of the invention, the subject in need thereof is suffering from a disease or condition such as, but not limited to respiratory and/or pulmonary disease or condition, male infertility, viral infection, a uterine disorder, an endometrial disorder or condition, cancer, primary cancer and/or metastatic cancer.

In some embodiments, a subject in need thereof refers to a subject with a pulmonary condition having clinically abnormal spirometry values. Examples of spirometry parameters which can indicate the need of a subject include, but are not

restricted to forced expiration volume (FEV_i), forced vital capacity (FVC), forced expiratory flow (FEF₂₅₋₇₅) and the like. In some embodiments of the invention administration of the rhDNase to the subject results in an improvement in one or more of the spirometric parameters.

5 Pulmonary administration of DNase

Pulmonary administration may be accomplished by suitable means known to those in the art. Pulmonary administration of DNase requires dispensing of the biologically active substance from a delivery device into the oral cavity of a subject during inhalation. For purposes of the present invention, compositions comprising DNase are administered via inhalation of dry powder formulation of the invention, via a dry powder inhaler delivery device. Such delivery devices are well known in the art and include, but are not limited to, metered dose and pre-metered dry powder inhalers, or any other appropriate delivery mechanisms that allow for dispensing of a solid or dry powder form.

15 Dry Powder Inhaler (DPI) Devices

According to some aspects of one embodiment, the dry powder formulation comprising DNase, or biologically active portion thereof, is delivered to a subject through a dry powder inhaler (DPI). A DPI is used to deliver an agent, such as DNase, in a solid or dry powder form using a subject's inspiration to deliver the dry powder to the lungs, instead of a mist. A DPI is used to breathe in (inhale) the DNase so that it goes directly into the subject's lungs. A DPI is a propellant-free device, wherein the agent to be delivered is blended with suitable carriers known in the art. The unit dose of agent used in a DPI device is often a dry powder blister disc or hard capsule. A DPI produces dispersible and stable dry powder formulations which are inhaled, including spray drying, spray-freeze drying, and micronized milling formulations. DPI devices have been used to deliver macromolecular agents, including insulin, interferon (IFN), and growth hormone (GH). Examples of DPI devices include, but are not limited to, the following:

The AIR® inhaler (Alkermes) which includes a small, breath-activated system that delivers porous powder from a capsule (see WO 99/66903 and WO 00/10541). The porous particles have an aerodynamic diameter of 1-5 μm and are prepared by spray drying. The AIR™ inhaler has been used to deliver albuterol, epinephrine, insulin, and

hGH. The TurboHaler® (AstraZeneca) is also a DPI which may be used in the methods of the invention and is described in EP patent 0799067, incorporated by reference herein. This DPI device is an inspiratory flow-driven, multidose dry-powder inhaler with a multi-dose reservoir that provides up to 200 doses of the drug formulation and dose ranges from a few micrograms to 0.5 mg. Examples of the TurboHaler™ include
5 Pulmicort® (also Pulmicort® TurbuHaler®), Oxis® (formoterol) and Symbicort® (budesonide/formoterol).

Eclipse™ (Aventis) represents a breath actuated reusable capsule device capable of delivering up to 20 mg of formulation. The powder is sucked from the capsule into a vortex chamber where a rotating ball aids in powder disaggregation as the
10 subject inhales (see U.S. Pat. No. 6,230,707 and WO9503846).

Another DPI device which may be used in the methods and compositions of the invention includes the Ultrahaler® (Aventis), as described in U.S. Pat. No. 5,678,538 and WO2004026380.

15 Another DPI device, which may be used in the methods and compositions of the invention includes the Bang Olufsen breath actuated inhaler, which is a disposable breath actuated inhaler using blister strips with up to sixty doses (see EP 1522325).

An active DPI (also usable as an MDI—described below) described in WO 94/19042 (Bespak) employs multiple, carbon fiber brush, setaceous electrodes to disperse powders and aerosols into fine/particles/mists. As the patient inhales, 1 to 10
20 kvolts is passed through the electrodes to disperse the powder/aerosol. A breath sensor is employed to initiate the electric discharge.

The HandiHaler® (Boehringer Ingelheim GmbH) is a single dose DPI device, which can deliver up to 30 mg of formulated drug in capsules (see WO2004024156).
25 An example of this device is Spiriva® (tiotropium bromide). The PADD DPI (Britannia Pharmaceuticals) is a pressurized aerosol dry powder delivery device utilizing a novel formulation comprised of surface active phospholipids, dipalmitoyl phosphatidyl choline (DPPC) and phosphatidyl glycerol (PG), prepared in the form of a fine powder. The PADD device offers the highest payload possible with a propellant
30 powered device, (see U.S. Pat. No. 6,482,391). Another DPI device, which may be used in the methods and compositions of the invention includes the Pulvinal® inhaler (Chiesi) which is a breath-actuated multidose (100 doses) dry powder inhaler (see U.S.

Pat. No. 5,351,683). The Pulvinal inhaler has been used to deliver respiratory drugs such as salbutamol (Butovent® Pulvinal®), beclomethasone (Clenil® Pulvinal®) as well as budesonide and formoterol.

Another DPI device which may be used in the methods and compositions of the invention includes NEXT DPITM, which features multidose capabilities, moisture protection, dose counting and doses only when proper aspiratory flow is reached (see 5 EP1 196146, U.S. Pat. No. 6,528,096, WO0178693, WO0053158).

The DirectHaler™ (Direct-Haler A/S) may also be used in the methods and compositions of the invention (see U.S. Pat. No. 5,797,392). This single dose, premeasured, pre-filled, disposable DPI device made from polypropylene resembles a 10 straw, and has been used to deliver formulations of budesonide and formoterol. The Accuhaler/Diskus™ (GlaxoSmithKline) is a disposable small DPI device using doses in double foil blister strips (see GB2242134), which has been used to deliver flutacasonone propionate/salmeterol xinafoate, flutacasonone propionate, salmeterol xinafoate, and salbutamol. 15

In addition, the methods may include the FlowCaps® (Hovione), a capsule-based, re-fillable, reusable, pen-shaped, moisture-proof passive dry-powder inhaler (see U.S. Pat. No. 5,673,686).

In one embodiment, the DPI device used in the invention is a multi-dose device 20 such as the Clickhaler® (Innovata PLC), (see U.S. Pat. No. 5,437,270), used to treat asthma and COPD with a variety of drugs, including salbutamol (Asmasal®), beclomethasone (Asmabec®), and procaterol hydrochloride (Meptin®) as well as budesonide and formoterol. Another DPI device suitable for use with the invention includes the Duohaler® (Innovata PLC) (see WO0139823). Duohaler® is actually 25 ideally suited for the delivery of fixed combination therapy with additional compositions/drugs for CF, asthma, COPD and the like.

In one embodiment, the DPI device used in the invention is an S2 unit dose (Innovata PLC), which is a re-useable or disposable single-dose DPI for the delivery of a wide range of therapeutics in high concentrations (see AU3320101).

30 Yet another DPI device which may be used in the methods and compositions of the invention includes Taifun® DPI (LAB International) which is a multiple-dose (up to 200) DPI device that is breath actuated and flow rate independent (see U.S. Pat. No.

6,132,394). In one embodiment, the DPI device used in the invention is MedTone® (Mannkind Corp., see WOO107107) which comprises an intake section, a mixing section, and a mouthpiece. The mouthpiece is connected by a swivel joint to the mixing section. The intake chamber comprises a piston with a tapered piston rod and spring, and one or more bleedthrough orifices to modulate the flow of air through the device. 5 The mixing section holds a capsule with holes containing a dry powder medicament, and further opens and closes the capsule when the intake section is at a certain angle to the mouthpiece. The mixing section is a Venturi chamber to impart a cyclonic flow to air passing through the mixing chamber. The mouthpiece includes a tongue depressor, and a protrusion to contact the lips of the user to tell the user that the DPI is in the correct position. Technosphere® Insulin System, used for the treatment of diabetes, consists of a dry-powder Technosphere® formulation (see US2004096403) of insulin and MedTone® inhaler through which the powder is inhaled into the deep lung. The powder formulation of the drug to be delivered in microparticles has a size range 15 between 0.5 and ten microns, preferably in the range of two to five microns, formed of a material releasing drug at a pH of greater than 6.4.). In the Technosphere device, a dry powder insulin formulation containing insulin complexed to 3,6-di(fumaryl4-aminobutyl)-2,5-diketopiperazine (hereinafter fumaryl diketopiperazine or FDKP) is used. The use of diketopiperazines for drug delivery is known in the art (see for example U.S. Pat. Nos. 5,352,461; U.S. Pat. No. 5,503,852; U.S. Pat. No. 6,071,497; 20 and U.S. Pat. No. 6,331,318). Pulmonary drug delivery using diketopiperazine and other microparticles is disclosed in U.S. Pat. No. 6,428,771. Particularly advantageous devices for powder delivery are disclosed in U.S. Patent No. 7,464,706 and in U.S. Pat. No. 6,923,175.

25 Another DPI device which may be used in the methods and compositions of the invention includes Xcelovair™ (Meridica/Pfizer) which features pre-metered, hermetically sealed doses in a fine particle fraction delivery to achieve up to 50% fine particle mass.

30 Yet another DPI device which may be used in the methods and compositions of the invention includes MicroDose® DPI (Microdose Technologies) which is a small electronic DPI device that uses piezoelectric vibrator (ultrasonic frequencies) to deaggragate the drug powder (small or large molecules, neat chemical or mixtures of

drug and lactose up to 3 mg drug) in an aluminum blister (single or multiple dose) (see U.S. Pat. No. 6,026,809).

In another embodiment, the DPI device used in the invention is Nektar Pulmonary Inhaler® (Nektar) which creates an aerosol cloud suitable for deep lung delivery (see AU4090599, U.S. Pat. No. 5,740,794), using compressed gas to aerosolize the powder. The Nektar Pulmonary Inhaler® is used in Exubera® inhalable insulin (Pfizer, Sanofi-Aventis, and Nektar), as well as to administer tobramycin, leuprolide, and single chain antibodies.

Also included in the invention is the Nektar Dry Powder Inhaler® (Nektar) which is used in combination with Nektar Pulmonary Technology® (see US2003094173). The Nektar DPI is ideal for large payloads (2-50 mg) and a variety of molecular sizes, and has been used to deliver tobramycin inhalation powder for lung infections in Cystic Fibrosis and amphotericin B for treatment of fungal infection. Also included in the invention is the active DPI Oriel™ (see WOO 168 169).

In addition, EasyHaler® (Orion Pharma), a multidose dry powder inhaler for lung and nasal delivery may be used in the methods and compositions of the invention (see WO02102444). The EasyHaler® includes Beclomet EasyHaler®/Atomide EasyHaler® (beclomethasone dipropionate) and Buventol EasyHaler®/Salbu EasyHaler® (salbutamol).

Also included in the invention is the Jethaler® (Pulmotec) which utilizes the MAG (mechanical aerosol generation from a highly compressed solid) technology for CFC-free dry-powder inhalation. The JetHaler® has been used to deliver budesonide (Budesonidratiopharm®).

Yet another DPI device which may be used in the methods and compositions of the invention includes AccuBreathe™ single dose DPI (Respirics) (see WO03035137, U.S. Pat. No. 6,561,186). Also included in the invention is the AcuBreather™ multidose DPI (Respirics) which uses an aclar/ PVC moisture protected blister cartridge capable of holding 25-50 mg of powder (30 dose and 15 dose devices respectively) and are capable of holding and delivering two different drug formulations simultaneously (see U.S. Pat. No. 6,561, 186), using i-Point™ technology for drug release.

Also included in the invention is the Twisthaler® (Schering-Plough), capable of 14-200 actuations (U.S. Pat. No. 5,829,434), packaged with a desiccant. Products including this DPI device include the Asmanex Twisthaler (mometasone furoate).

Another DPI device which may be used in the methods and compositions of the invention includes the multidose SkyeHaler® DPI (SkyePharma) (see U.S. Pat. No. 6,182,655, WO97/20589), for dosing from 200 ug to 5 mg. This DPI is device is included in Foradil Certihaler® (formoterol fumarate). Also included in the invention is the refiUable, multidose Novolizer® (Meda AB) dry powder inhaler (U.S. Pat. No. 5,840, 279, U.S. Pat. No. 6,071,498, WO9700703).

Another DPI device which may be used in the methods and compositions of the invention includes the Blister Inhaler™ (Meda AB), which is a refiUable, multi-dose, breath activated, dry powder inhaler with dose counter (U.S. Pat. No. 5,881,719, WO9702061), able to deliver moisture-sensitive compounds (e.g. proteins and peptides).

Other DPI devices include the SpinHaler® (Aventis and Rhone-Poulenc Rorer); the unit dose DPI (Bespak; a single unit dose device; see US Pat. No. US6945953), the DiskHaler® (GlaxoSmithKline; a multidose device for local lung delivery—see U.S. Pat. No. 5,035,237), Rotahaler® (GlaxoSmithKline) (see U.S. Pat. No. 5,673,686, U.S. Pat. No. 5,881,721); LABHaler® (LAB International; a breath-actuated disposable single dose dry powder delivery device); AirMaXTM (Ivax; a multiple dose reservoir inhaler; see U.S. Pat. No. 5,503,144); Aerolizer™ (Novartis); see U.S. Pat. No. 6,488,027, U.S. Pat. No. 3,991, 761); Rexam DPI (Rexam Pharma; see U.S. Pat. No. 5,651, 359 and EP0707862; bead inhaler multiple dose (Valois; WO0035523, U.S. Pat. No. 6,056,169; a multiple dose DPI pulmonary delivery device on license from Elan/Dura/Quadrant); Aspirair® (Ventura; WO 02/089880; a single dose, breath activated DPI); and Gyrohaler® (Ventura; GB2407042; a passive disposable DPI). Other examples of commercially available dry powder inhalers suitable for use in accordance with the methods herein include the Spinhaler® powder inhaler (Fisons) and the Ventolin® Rotahaler® (GlaxoSmithKline). See also the dry powder delivery devices described in WO 93/00951, WO 96/09085, WO 96/32152, and U.S. Pat. Nos. 5,458,135, 5,785,049, and 5,993,783, herein incorporated by reference.

In one embodiment, the invention provides a dry powder inhaler (DPI) device for pulmonary administration of DNase to a subject, wherein the DPI device comprises a reservoir comprising an inhalable powder or dry powder composition comprising the DNase, and a means for introducing the inhalable powder or dry powder composition into the subject via inhalation. The invention also provides an inhalable powder which
5 comprises the DNase and is administered to the subject via a dry powder inhaler (DPI).

The DPI device used in the invention may be either a single dose or a multidose inhaler. In addition, the DPI device used in the invention may also be either pre-metered or device-metered.

10 Metered Dose Inhaler (MDI) Device

In one embodiment, the DNase, including an enzymatically active portion thereof, is delivered to a subject through metered dose inhaler (MDI) device. An MDI device uses a propellant to deliver reproducible metered drug dose to the lung and/or airways, and comprises a drug or agent, propellants (e.g. hydrofluoroalkanes (HFA)),
15 surfactants (e.g. phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositol, lysophosphatidyl choline, phosphatidic acid, triglycerides, monoglycerides, soy lecithin, fatty acids, and alkyl-polyglycosides), and solvents. An MDI device is often a compact pressurized dispenser, including a canister, metering valve, and spacer. The dose administered by an MDI device is generally in mg and ranges in volume from
20 about 25 to 100 mL. Additionally, MDI devices are advantageous as they are tamper-proof.

Examples of CFC-free MDI products include Albuterol® HFA (Ivax), Atrovent®-HFA (Boehringer-Ingelheim), Proventil®-HFA (3M), Flovent®-HFA (GSK), Qvar® (3M), Ventolin® HFA(GSK), Xopenex® HFA (3M/Sepracor), Salamol
25 Easi-Breathe® CFC-Free (Ivax), Berotec® (Boehringer-Ingelheim), Berodual® (Boehringer-Ingelheim), Intal® Forte (Rhone/Aventis), and Seretide® EvoHaler® (GSK).

Examples of MDI devices include, but are not limited to, the following:

In one embodiment, the invention provides an MDI device for pulmonary
30 administration of DNase to a subject, wherein the MDI device is anAutoHaler® (3M) (see U.S. Pat. No. 6,120,752). Examples of AutoHaler® devices being used to deliver therapeutic agents include Aerobid® (flunisolide), Alupent® (metaproterenol sulphate),

Atrovent®/Atovent®-HFA (ipratropium bromide), Combivent® (albuterol sulfate/ipratropium bromide), MaxAir® AutoHaler® (pirbuterol acetate), Proventil®-HFA (albuterol sulphate), Qvar® (beclomethasone dipropionate) and Xopenex® HFA (levalbuterol hydrochloride).

5 Another MDI device which may be used in the methods and compositions of the invention includes the breath-activated MD Turbo™ (Accentia Bio), which transforms metered-dose inhalers into a breath-activated, dose-counting inhaler.

In one embodiment, the invention provides an MDI device for pulmonary administration of DNase to a subject, wherein the MDI device is the continuous
10 inhalation flow device WatchHaler® (Activaero GmbH).

The portable drug delivery system EZ Spacer® (AirPharma) may also be used in the methods and compositions of the invention. In another embodiment, the Asmair® (Bang and Olufsen Medicom AS) MDI. In yet another embodiment, the invention includes an Active DPI/MPI device (Bespak) (see W094 19042). In still another
15 embodiment, the invention provides an MDI device for pulmonary administration of DNase to a subject, wherein the MDI device is a device for delivering metered aerosols comprising an active ingredient in solution in a propellant consisting of a hydrofluoroalkane (HFA) (see WOO 149350; Chiesi).

Other examples of MDI devices which may be used in the invention include
20 MDI inhalers described in U.S. Pat. No. 6,170,717 (GlaxoSmithKline); EasiBreath® MDI (Ivax; W0193933, U.S. Pat. No. 5,447,150); MDI breath coordinated inhaler and breath actuated inhaler (Kos; CA2298448 and WO2004082633); Tempo™ (MAP Pharma; U.S. Pat. No. 6,095,141, U.S. Pat. No. 6,026, 808 and U.S. Pat. No. 6,367,471); Xcelovent™ (Meridica/Pfizer; W09852634; a breath operated device that
25 also has a dose counter feature); and Increased dosage MDI (Nektar see WO2004041340; a device capable of delivering 2 mg to 5 mg of a formulated drug using HFA propellants) and a MDI described in WO03053501 (Vectura).

Thus, the invention also includes a metered dose inhaler (MDI) device for pulmonary administration of DNase to a subject, the MDI device comprising a
30 pressurized canister comprising an aerosol comprising the DNase and a propellant, and a means for introducing the aerosol into the subject via inhalation.

Formulations

DNase for use in the methods of the invention is formulated in dry powder formulation suitable for inhalation. Suitable preparations include all dry powder formulation preparations so long as the particles comprising the protein composition are delivered in a size range consistent with that described for the delivery device, e.g., a
5 dry powder form of the formulation.

Thus, a liquid formulation comprising DNase, or enzymatically active portion thereof, intended for use in the methods of the present invention may either be used as a liquid solution or suspension in the delivery device or first be processed into a dry
10 powder form using lyophilization or spray-drying techniques well known in the art. Powder comprising a DNase such as a plant expressed recombinant human DNase I, may also be prepared using other methods known in the art, including crystallization or precipitation (see, for example, dry powder microspheres (PROMAXX; Baxter) described in U.S. Pat. No. 5,525,519; U.S. Pat. No. 5,599, 719; U.S. Pat. No. 5,578,709;
15 U.S. Pat. No. 5,554,730; U.S. Pat. No. 6,090,925; U.S. Pat. No. 5,981,719; U.S. Pat. No. 6,458,387, each of which is incorporated herein by reference).

Where the liquid formulation is lyophilized prior to use in the delivery methods of the invention, the lyophilized composition may be milled to obtain the finely divided dry powder consisting of particles within the desired size range noted above. Where
20 spray-drying is used to obtain a dry powder form of the liquid formulation, the process is carried out under conditions that result in a substantially amorphous finely divided dry powder consisting of particles within the desired size range noted above. Similarly, if the starting formulation is already in a lyophilized form, the composition can be milled to obtain the dry powder form for subsequent preparation as an aerosol or other
25 preparation suitable for pulmonary inhalation. Where the starting formulation is in its spray-dried form, the composition has preferably been prepared such that it is already in a dry powder form having the appropriate particle size for dispensing as an aqueous or nonaqueous solution or suspension or dry powder form in accordance with the pulmonary administration methods of the invention. For methods of preparing dry
30 powder forms of formulations, see, for example, WO 96/32149, WO 97/41833, WO 98/29096, and U.S. Pat. Nos. 5,976,574, 5,985,248, and 6,001,336; herein incorporated by reference.

The resulting dry powder form of the composition is then placed within an appropriate delivery device for subsequent preparation as an aerosol or other suitable preparation that is delivered to the subject via pulmonary inhalation. Where the dry powder form of the formulation is to be prepared and dispensed as an aqueous or non-aqueous solution or suspension, a metered-dose inhaler, or other appropriate delivery device is used. A pharmaceutically effective amount of the dry powder form of the composition is administered in an aerosol or other preparation suitable for pulmonary inhalation. The amount of dry powder form of the composition placed within the delivery device is sufficient to allow for delivery of a pharmaceutically effective amount of the composition to the subject by inhalation. Thus, the amount of dry powder form to be placed in the delivery device will compensate for possible losses to the device during storage and delivery of the dry powder form of the composition. Following placement of the dry powder form within a delivery device, the properly sized particles as noted above are suspended in an aerosol propellant. The pressurized nonaqueous suspension is then released from the delivery device into the air passage of the subject while inhaling. The delivery device delivers, in a single or multiple fractional dose, by pulmonary inhalation a pharmaceutically effective amount of the composition to the subject's lungs. The aerosol propellant may be any conventional material employed for this purpose, such as a chlorofluorocarbon, a hydrochlorofluorocarbon, a hydrofluorocarbon, or a hydrocarbon, including trichlorofluoromethane, dichlorodifluoro-methane, dichlorotetrafluoromethane, dichlorodifluoro-methane, dichlorotetrafluoroethanol, and 1,1,1,2-tetra-fluoroethane, or combinations thereof. A surfactant may be added to the formulation to reduce adhesion of the protein-containing dry powder to the walls of the delivery device from which the aerosol is dispensed. Suitable surfactants for this intended use include, but are not limited to, sorbitan trioleate, soya lecithin, and oleic acid. Devices suitable for pulmonary delivery of a dry powder form of a protein composition as a nonaqueous suspension are commercially available. Examples of such devices include the Ventolin metered-dose inhaler (Glaxo Inc., Research Triangle Park, N.C.) and the Intal Inhaler (Fisons, Corp., Bedford, Mass.). See also the aerosol delivery devices described in U.S. Pat. Nos. 5,522,378, 5,775,320, 5,934,272 and 5,960,792, herein incorporated by reference.

Where the solid or dry powder form of the formulation is to be delivered as a dry powder form, a dry powder inhaler or other appropriate delivery device is preferably used. The dry powder form of the formulation is preferably prepared as a dry powder aerosol by dispersion in a flowing air or other physiologically acceptable gas stream in a conventional manner. Examples of dry powder inhalers suitable for use
5 in accordance with the methods herein are described above.

When a formulation comprising a DNase is processed into a solid or dry powder form for subsequent delivery as an aerosol, it may be desirable to have carrier materials present that serve as a bulking agent or stabilizing agent. In this manner, the present
10 invention discloses stabilized lyophilized or spray-dried formulations comprising DNase for use in the methods of the present invention. These compositions may further comprise at least one bulking agent, at least one agent in an amount sufficient to stabilize the protein during the drying process, or both. By "stabilized" is intended the DNase thereof retains its monomeric or multimeric form as well as its other key
15 properties of quality, purity, and potency following lyophilization or spray-drying to obtain the solid or dry powder form of the composition.

Preferred carrier materials for use as a bulking agent include glycine, mannitol, alanine, valine, or any combination thereof, most preferably glycine. The bulking agent is present in the formulation in the range of 0% to about 10% (w/v), depending upon the
20 agent used. When the bulking agent is glycine, it is present in the range of about 0% to about 4%, preferably about 0.25% to about 3.5%, more preferably about 0.5% to 3.0%, even more preferably about 1.0% to about 2.5%, most preferably about 2.0%. When the bulking agent is mannitol, it is present in the range of about 0% to about 5.0%, preferably about 1.0% to about 4.5%, more preferably about 2.0% to about 4.0%, most
25 preferably about 4.0%. When the bulking agent is alanine or valine, it is present in the range of about 0% to about 5.0%, preferably about 1.0% to about 4.0%, more preferably about 1.5% to about 3.0%, most preferably about 2.0%.

Preferred carrier materials for use as a stabilizing agent include any sugar or sugar alcohol or any amino acid. Preferred sugars include sucrose, trehalose, raffinose,
30 stachyose, sorbitol, glucose, lactose, dextrose or any combination thereof, preferably sucrose. When the stabilizing agent is a sugar, it is present in the range of about 0% to about 9.0% (w/v), preferably about 0.5% to about 5.0%, more preferably about 1.0% to

about 3.0%, most preferably about 1.0%. When the stabilizing agent is an amino acid, it is present in the range of about 0% to about 1.0% (w/v), preferably about 0.3% to about 0.7%, most preferably about 0.5%. These stabilized lyophilized or spray-dried compositions may optionally comprise methionine, ethylenediaminetetracetic acid (EDTA) or one of its salts such as disodium EDTA or other chelating agent, which protect DNase against methionine oxidation. Methionine is present in the stabilized lyophilized or spray-dried formulations at a concentration of about 0 to about 10.0 mM, preferably about 1.0 to about 9.0 mM, more preferably about 2.0 to about 8.0 mM, even more preferably about 3.0 to about 7.0 mM, still more preferably about 4.0 to about 6.0 mM, most preferably about 5.0 mM. EDTA is present at a concentration of about 0 to about 10.0 mM, preferably about 0.2 mM to about 8.0 mM, more preferably about 0.5 mM to about 6.0 mM, even more preferably about 0.7 mM to about 4.0 mM, still more preferably about 0.8 mM to about 3.0 mM, even more preferably about 0.9 mM to about 2.0 mM, most preferably about 1.0 mM.

The composition of the invention can be formulated with addition ingredients. These can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel™, or corn starch; a lubricant such as magnesium stearate or Sterotes™; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring. When the dosage unit form is a capsule, it can contain, in addition to material of the above type, a liquid carrier. In addition, dosage unit forms can contain various other materials which modify the physical form of the dosage unit, for example, coatings of sugar, shellac, or other enteric agents.

The stabilized lyophilized or spray-dried compositions may be formulated using a buffering agent, which maintains the pH of the formulation within an acceptable range when in a liquid phase, such as during the formulation process or following reconstitution of the dried form of the composition. In some embodiments the pH is in the range of about pH 4.0 to about pH 8.5, about pH 4.5 to about pH 7.5, about pH 5.0 to about pH 6.5, about pH 5.6 to about pH 6.3, and about pH 5.7 to about pH 6.2. Suitable pH's include about 4.0, about 4.5, about 5.0, about 5.1, about 5.2, about 5.3,

about 5.4, about 5.5, about 5.6, about 5.7, about 5.8, about 5.9, about 6.0, about 6.1, about 6.2, about 6.3, about 6.4, about 6.5, about 6.6, about 6.7, about 6.8, about 6.9, about 7.0, about 7.1, about 7.2, about 7.3, about 7.4, about 7.5, about 7.7, about 7.8, about 7.9, about 8.0, about 8.2, about 8.4, about 8.6, about 8.8, about 9.0. In one particular embodiment, the pH is about 7.0 to 8.2. Suitable buffering agents include, but are not limited to, citrate buffer, phosphate buffer, succinate buffer, more particularly a sodium citrate/citric acid. Alternatively imidazole or histidine or other base/acid that maintains pH in the range of about pH 4.0 to about 8.5 can be used. Buffers are chosen such that they are compatible with the drying process and do not affect the quality, purity, potency, and stability of the protein during processing and upon storage.

Any of the formulations comprising human DNase I contemplated for use in the methods of the invention may be formulated with at least one surfactant. For pulmonary intracellular administration of the DNase, the surfactant can be in an amount sufficient to enhance absorption of the inhaled particles comprising DNase to obtain an absorbable composition for use in pulmonary inhalation in accordance with the methods described herein. Any surfactant that enhances absorption of a formulation comprising DNase thereof in the manner disclosed herein may be used to obtain these absorbable protein-containing formulations. Surfactants suitable for use in enhancing absorption of the inhaled DNase include, but are not limited to, polyoxyethylene sorbitol esters such as polysorbate 80 (Tween 80) and polysorbate 20 (Tween 20); polyoxypropylene-polyoxyethylene esters such as Poloxamer 188; polyoxyethylene alcohols such as Brij 35; a mixture of polysorbate surfactants with phospholipids such as phosphatidylcholine and derivatives (dipalmitoyl, dioleoyl, dimyristyl, or mixed derivatives such as 1-palmitoyl, 2-oleoyl, etc.), dimyristolglycerol and other members of the phospholipid glycerol series; lysophosphatidylcholine and derivatives thereof; mixtures of polysorbates with lysolecithin or cholesterol; a mixture of polysorbate surfactants with sorbitan surfactants (such as sorbitan monoleate, dioleate, trioleate or others from this class); poloxamer surfactants; bile salts and their derivatives such as sodium cholate, sodium deoxycholate, sodium glycodeoxycholate, sodium taurocholate, etc.; mixed micelles of DNase with bile salts and phospholipids; Brij surfactants (such as Brij 35-PEG923) lauryl alcohol, etc.). The amount of surfactant to be added is in the range of

about 0.005% to about 1.0% (w/v), preferably about 0.005% to about 0.5%, more preferably about 0.01% to about 0.4%, even more preferably about 0.03% to about 0.3%, most preferably about 0.05% to about 0.2%.

The formulation of the invention may include a suitable dosage according to the disorder being treated. In one embodiment, the formulation of the invention comprises a dose of about 0.01 mg to 10 mg of DNase or enzymatically active portion thereof. Alternatively, the formulation of the invention comprises a dose of about 0.1 mg to 5 mg; about 1 mg to 5 mg; about 2.5 mg to 5 mg, about 2.0 to 4.5 mg, about 2.2 to 4.0 mg, about 2.0 to 3.0 mg, about 2.2 to 3.0 mg, about 2.3 to 3.0 mg, about 2.4 to 2.8 mg, about 2.4 to 2.6 mg; or about 2.5 mg of the DNase or enzymatically active portion thereof. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition.

Thus, in some embodiments, the dosage regimen includes, but is not limited to a single dose of the dry powder formulation of the invention, of 1.0 to 10 mg DNase I, administered daily, a single dose of 2.0 to 5 mg DNase I, administered daily, a single dose of 2.0- 3.0 mg DNase I, administered daily, a plurality of doses, each dose comprising 1.0-3.0 mg DNase, the doses administered at least twice, 2-3 times, 2-4 times or 2-6 times daily, a plurality of doses, each dose comprising 1.0-3.0 mg DNase, the doses administered once every 36 hours, once every 36-48 hours, once every 36-72 hours, once every 2-3 days, once every 2-4 days, once every 2-5 days, or once every week, a plurality of doses, each dose comprising 1.0-3.0 mg DNase, the doses administered once every 36 hours, once every 36-48 hours, once every 36-72 hours, once every 2-3 days, once every 2-4 days, once every 2-5 days, or once every week.

Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The formulation can be formulated as a solution, microemulsion, dispersion, liposome, or other ordered structure suitable to high drug concentration before processing into a dry powder. Sterile inhalable solutions can be prepared by incorporating the active compound (i.e., rhDNase, DNase I or other DNase) in the required amount in an appropriate solvent with one or a combination of

ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. The proper fluidity of a solution can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prolonged action of inhalable compositions can be brought about by including in the composition an agent that delays absorption, for example, monostearate salts and gelatin.

In one embodiment, the DNase or active portion for use in the methods of the invention is incorporated into a pharmaceutical formulation as described in Examples 2-5. Supplementary active compounds can also be incorporated into the compositions for pulmonary delivery. In certain embodiments, a DNase or active portion for use in the methods of the invention is coformulated with and/or coadministered with one or more additional therapeutic agents mentioned hereinabove. For example, DNase I or active portion of the invention may be coformulated and/or coadministered with one or more additional compositions that reduce actin inhibition (e.g. magnesium or potassium salts), and/or one or more chemical agents that inhibit mucus production (such as anti-inflammatory agents, bronchodilators and/or mucus secretion blockers, as described in US 7,763,610) or any combination thereof. Furthermore, the DNase of the invention may be used in combination with two or more of the foregoing therapeutic agents. Such combination therapies may advantageously utilize lower dosages of the administered therapeutic agents, thus avoiding possible side effects, complications or low level of response by the patient associated with the various monotherapies.

The formulations of the invention may include a "therapeutically effective amount" or a "prophylactically effective amount" of a DNase protein or active portion of the invention. A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result. A therapeutically effective amount of the DNase may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the DNase or active portion thereof to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the DNase are outweighed by the therapeutically beneficial effects. A "prophylactically effective

amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount will be less than the therapeutically effective amount.

5 The invention also pertains to packaged formulations or kits for pulmonary administration of a DNase, e.g., DNase I. In one embodiment of the invention, the kit comprises a DNase, such as DNase I, and instructions for pulmonary administration of the DNase, wherein the DNase is in a dry powder formulation suitable for inhalation. The instructions may describe when, e.g., at day 1, day 4, week 0, week 2, week 4, etc.,
10 the different doses of DNase shall be administered via inhalation to a subject for treatment.

 Another aspect of the invention pertains to kits containing a dry powder formulation comprising a DNase, such as DNase I, and a pharmaceutically acceptable carrier, and one or more formulations each comprising an additional therapeutic agent,
15 and a pharmaceutically acceptable carrier.

 The package or kit alternatively can contain the DNase and it can be promoted for use, either within the package or through accompanying information, for the uses or treatment of the disorders described herein. The packaged formulations or kits further can include a second agent (as described herein) packaged with or copromoted with
20 instructions for using the second agent with a first agent (as described herein).

 The term "tissue" refers to part of an organism consisting of cells designed to perform a function or functions. Examples include, but are not limited to, brain tissue, retina, skin tissue, hepatic tissue, pancreatic tissue, bone, cartilage, connective tissue, blood tissue, muscle tissue, cardiac tissue brain tissue, vascular tissue, renal tissue,
25 pulmonary tissue, gonadal tissue, hematopoietic tissue.

 For any preparation used in the methods of the invention, the therapeutically effective amount or dose can be estimated initially from in vitro and cell culture assays. For example, a dose of the dry powder formulation can be formulated in animal models to achieve a desired concentration or titer. Such information can be used to more
30 accurately determine useful doses in humans.

 Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures in vitro, in cell cultures or

experimental animals. The data obtained from these in vitro and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g., Fingl, et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1).

Dosage amount and interval may be adjusted individually, for example, to provide serum and cell levels of the active ingredient which are sufficient to induce or suppress the biological effect (minimal effective concentration, MEC). The MEC will vary for each preparation, but can be estimated from in vitro data. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. Detection assays can be used to determine plasma concentrations.

Depending on the severity and responsiveness of the condition to be treated, dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks or until cure is effected or diminution of the disease state is achieved.

The amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc.

Compositions of some embodiments of the invention may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert. Compositions comprising a preparation of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an

appropriate container, and labeled for treatment of an indicated condition, as is further detailed above.

The formulation can be used for treatment or prevention of a mucus-related respiratory condition in a subject in need thereof. Thus, according to another aspect of the present invention there is provided a method for treating or preventing a respiratory condition such as cystic fibrosis (CF) or COPD in a subject in need thereof, the method comprising administering to the subject an effective amount of a formulation which includes, as an active ingredient thereof, a human DNase I protein, and a pharmaceutical acceptable carrier. In some embodiments of the present invention, the human DNase I protein has an N-terminal Glycine residue. In other embodiments, the human DNase I protein has an amino acid sequence as set forth in SEQ ID NO: 5.

According to some embodiments of the present invention, the dry powder formulation comprising the human DNase I is administered by pulmonary administration, as detailed hereinabove.

In yet other embodiments, DNase can be used for treatment or prevention of male infertility, endometrial and uterine inflammatory disorders, viral infections and cancer, in particular, metastatic growth. In some embodiments, treatment of infertility, endometrial and uterine inflammatory disorders, viral infections and cancer metastases is effected by administering to a subject in need thereof an effective amount of the dry powder formulation comprising the DNase I via a mode of pulmonary administration providing systemic delivery of the human recombinant DNase. In some embodiments, administering the dry powder formulation comprising the DNase I via pulmonary administration results in reduction in extracellular DNA in the subject, for example, of the extracellular DNA in a secretion, fluid or tissue of the subject. In some embodiments, the extracellular DNA is pathological, and can be associated with a disease or disorder from which the subject suffers.

The term "treating" refers to inhibiting, preventing or arresting the development of a pathology (disease, disorder or condition) and/or causing the reduction, remission, or regression of a pathology. Those of skill in the art will understand that various methodologies and assays can be used to assess the development of a pathology, and similarly, various methodologies and assays may be used to assess the reduction, remission or regression of a pathology.

As used herein, the term "preventing" refers to keeping a disease, disorder or condition from occurring in a subject who may be at risk for the disease, but has not yet been diagnosed as having the disease.

As used herein, the term "subject" includes mammals, preferably human beings
5 at any age which suffer from the pathology. Preferably, this term encompasses individuals who are at risk to develop the pathology.

As used herein the phrase "treatment regimen" refers to a treatment plan that specifies the type of treatment, dosage, schedule and/or duration of a treatment provided to a subject in need thereof (e.g., a subject diagnosed with a pathology). The selected
10 treatment regimen can be an aggressive one which is expected to result in the best clinical outcome (e.g., complete cure of the pathology) or a more moderate one which may relieve symptoms of the pathology yet results in incomplete cure of the pathology. It will be appreciated that in certain cases the more aggressive treatment regimen may be associated with some discomfort to the subject or adverse side effects (e.g., a damage to
15 healthy cells or tissue). The type of treatment can include a surgical intervention (e.g., removal of lesion, diseased cells, tissue, or organ), a cell replacement therapy, an administration of a therapeutic drug (e.g., receptor agonists, antagonists, hormones, chemotherapy agents) in a local or a systemic mode, an exposure to radiation therapy using an external source (e.g., external beam) and/or an internal source (e.g.,
20 brachytherapy) and/or any combination thereof. The dosage, schedule and duration of treatment can vary, depending on the severity of pathology and the selected type of treatment, and those of skills in the art are capable of adjusting the type of treatment with the dosage, schedule and duration of treatment.

It is expected that during the life of a patent maturing from this application many
25 relevant vectors, promoter elements, plant cells and carriers will be developed and the scope of the terms provided herein is intended to include all such new technologies *a priori*.

As used herein the term "about" refers to $\pm 10\%$.

The terms "comprises", "comprising", "includes", "including", "having" and
30 their conjugates mean "including but not limited to".

The term "consisting of" means "including and limited to".

The term "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the additional ingredients, steps and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure.

5 As used herein, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a compound" or "at least one compound" may include a plurality of compounds, including mixtures thereof.

Throughout this application, various embodiments of this invention may be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

Whenever a numerical range is indicated herein, it is meant to include any cited numeral (fractional or integral) within the indicated range. The phrases "ranging/ranges between" a first indicate number and a second indicate number and "ranging/ranges from" a first indicate number "to" a second indicate number are used herein interchangeably and are meant to include the first and second indicated numbers and all the fractional and integral numerals therebetween.

25 As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

30 As used herein, the term "treating" includes abrogating, substantially inhibiting, slowing or reversing the progression of a condition, substantially ameliorating clinical

or aesthetical symptoms of a condition or substantially preventing the appearance of clinical or aesthetical symptoms of a condition.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination
5 in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various
10 embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

15

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions illustrate some embodiments of the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant
20 DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley &
25 Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994);
30 "Culture of Animal Cells - A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition),

Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 5 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, 10 (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1, 2, 317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. 15 Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

***EXAMPLE I: CONSTRUCTION OF THE PLANT DEOXYRIBONUCLEASE I
EXPRESSION CONSTRUCT***

20 cDNA encoding the human deoxyribonuclease I (DNase I) protein (EC 3.1.21.1 GenBank: NM_005223) was optimized and synthesized by GENEART AG (Regensburg, Germany). The codon usage without the 22 amino acid leader peptide (e.g. endoplasmic reticulum target signal peptide) was adapted to the codon bias of 25 *Nicotiana tabacum* genes. During the optimization process the following cis-acting sequence motifs were avoided: Internal TATA-boxes, chi-sites and ribosomal entry sites, AT-rich or GC-rich sequence stretches, RNA instability elements ("killer motifs"), Repeat sequences and RNA secondary structures, splice donor (cryptic) and acceptor sites, branch points. In addition, regions of very high (>80%) or very low (<30%) GC 30 content were avoided.

The nucleotide sequence of the native human DNase I leader peptide (endoplasmic reticulum target signal peptide) (Figure 1, first 22 amino acids in red) of

the full length human DNase I protein (GenBank: NM_005223) was replaced with a nucleotide sequence encoding the 33 amino acid endoplasmic reticulum targeting signal peptide (leader peptide) of the Arabidopsis ABPI protein (marked in green in Figure 1, SEQ ID NO: 4). This ER targeting signal peptide provides efficient targeting of DNase I to the secretory pathway and is then cleaved from the polypeptide, by signal peptidase, once the protein has been translocated into the endoplasmic reticulum. In some cases the cleavage of ABPI signal is not complete and the last (C-terminal) amino acid Glycine of the signal peptide remains, being added to the N terminal of the mature DNase I protein. As the optimized DNase I coding sequence does not contain any compartment (organelle) localization signal at the 3' terminus, the recombinant DNase I, absent a C-terminal localization signal is directed to the plant cell apoplast (the extracellular space surrounding the plant cell symplast), and when expressed in plant cell cultures, is subsequently secreted into the culture medium. SEQ ID NO: 1 represents the complete coding sequence of the expressed recombinant human DNase I polypeptide, including the N-terminal ABPI endoplasmic reticulum targeting signal peptide (SEQ ID NO: 4) and the human DNase I protein (SEQ ID NO: 6).

EXAMPLE 2: EXPRESSION OF RECOMBINANT hDNase I IN PLANTS

Transient expression system in N.benthamiana

The use of plant viral vectors was chosen in this case as an alternative to transgenic plants, allowing for the rapid, high level transient expression of proteins in mature whole plants.

The protein of interest was expressed from a strong subgenomic viral coat protein promoter. The system relies on transient amplification (by agroinfection) of viral vectors delivered to a plant by *Agrobacterium*, in which the plant functional promoter and the cDNA encoding a viral replicon are transferred as T-DNA from *Agrobacterium* into plant cells. The T-DNA is transcribed *in-planta* by the plant promoter to generate biologically active viral RNA that initiates self replication.

For the transient expression a 3-vector recombination system based on the system previously developed as described (Gleba et al., Vaccine 23 2042-2048, 2005) was employed. DNase I cDNA was inserted into one of the vectors, and the two other vectors contained genes for construction of the whole viral replicon (RdRp and

Integrase), thus generating a biologically active viral RNA capable of initiating self replication.

Transfection of whole plants -

N. Benthamiana plants were germinated and grown in commercial mix soil
5 (Givaat Ada, IL) supplemented with granular slow release fertilizer (Scott Marysville, OH) under a long day (16h light / 8h dark) light regime at 24°C-25°C.

Agrobacteria were transformed with the pICH20866-DNaseI based replicon vector system using electroporation (2500V, 5msec) [den Dulk-Ra, A. and Hooykaas, P.J. (1995) *Methods Mol. Biol.* 55:63-72]. Plants were infiltrated with *Agrobacteria*
10 containing the 3 ICON plasmids by vacuum infiltration with standard methods known in the art. Briefly, *N. benthamiana* plants 5-6 week old were infiltrated by immersing all aerial plant organs into a bacterial suspension and were placed in a vacuum chamber. A minus (-) 0.8 bar vacuum was applied for 1 minute, followed by a quick return to atmospheric pressure. Plants were returned to the greenhouse for additional 5-7 days
15 under the same growth conditions.

Screening for targeting to plant cell compartments was accomplished using the 3 vector recombination system described above, with the DNase I-based vector including a sequence encoding an N-terminal Arabidopsis ABPI signal peptide (marked in green in Figure 1, SEQ ID NO: 4), followed by the nucleotide sequence of human DNase I,
20 and further followed by one of the below:

- 1) A nucleotide sequence (SEQ ID NO: 14) encoding the endoplasmic reticulum retention signal SEKDEL (SEQ ID 13), allowing retrieval of the expressed protein from the Golgi apparatus, effectively maintaining the protein in the endoplasmic reticulum;
- 25 2) A nucleotide sequence (SEQ ID NO: 16) encoding for the vacuole targeting signal peptide GLLVDTM (SEQ ID 15), allowing transfer of the expressed protein from the Golgi apparatus to the cell vacuole; or
- 3) No addition of any compartment localization signal at the 3' terminus of the nucleic acid sequence, directing localization of the expressed DNase I protein to the
30 plant cell apoplast, by default.

Tobacco Plant Extract:

Samples of *Nicotiana benthamiana* leaves were harvested 5 days post infiltration and extracted in Laemmli buffer for SDS-PAGE, or in extraction buffer (25mM HEPES-NaOH, 40mM CaCl₂, 40mM MgCl₂, pH 7.5) for assays of catalytic activity and ELISA of the plant expressed protein.

Tobacco Plant Extract Purification:

Human DNase I protein from plant extracts was purified by centrifugation of the homogenized plant mass, discarding the plant debris. The supernatant was heated to 60°C for 10 minutes and filtered through a 3 µm filter, followed by filtration through a 0.65 µm filter. The filtrate was further purified on a hydrophobic interaction chromatography resin (Butyl 650C, Toyopearl) followed by further purification on an anion exchange chromatography resin (Poros 50HQ). The final material was filtered for sterility (0.2 µm).

Stable expression in N. tabacum BY2 cells

Agrobacterium mediated transformation is widely used to introduce foreign genes into a plant cell genome. This technique is based on the natural capability of the agrobacterium to transform plant cells by transferring a plasmid DNA segment, the transferred DNA (T-DNA), into the host cell genome. Using this approach, a T-DNA molecule, consisting of a foreign gene and its regulatory elements, is randomly introduced into the plant genome. The site of integration, as well as the copy number of the gene insertions is not controlled, thus the transformation process results in a 'pool' of transgenic cells composed of cells with various levels of expression of the transgene. The transgenic 'pool' is subsequently used for clone isolation. Clone isolation results in the establishment of many single cell lines, from which the clone with the highest expression level of the foreign gene is then selected.

BY2 (Bright Yellow 2) suspension culture was co-cultivated, for 48 hours, with the *Agrobacterium tumefaciens* EHA105 strain carrying the vector harboring the DNase I gene and the neomycin phosphotransferase (NPTII) selection gene.

Subsequently, the cells were kept in media supplemented with 50mg/L of Kanamycin and 250mg/L Cefotaxime. The NPTII gene confers resistance to Kanamycin, thus only NPTII positive BY2 cells survive in this selection media. The Cefotaxime was used to selectively kill the agrobacterium, the plant cells being resistant

to this antibiotic. Once a nicely growing transgenic cell suspension was established, it was used for screening and isolating individual cell lines. To allow for the selection of individual cell lines, aliquots of highly diluted cell suspension were spread on solid BY-2 medium. The cells were then grown until small calli developed. Each callus was then re-suspended in liquid culture. Media was then sampled and evaluated for DNase I levels. The lines that secreted relatively high DNase I levels were then further re-analyzed and compared for DNase I levels ending with the final selection of candidate DNase I expressing lines.

Media samples of transformed BY2 cells expressing the human DNase I protein were collected and when required, concentrated 5x by centrifugal filters (Amicon Ultra, 10K, #UFC501096). DNase I catalytic activity in cell's media was determined by DNA-Methyl Green assay and compared to total DNase I amount, determined by Enzyme-linked immunosorbent assay (ELISA 1) as described hereinbelow.

Protein Purification from BY-2 cells:

Recombinant human DNase-I protein secreted from tobacco suspension plant cells was purified by the following steps: at the end of the fermentation the intact tobacco cells were separated from the media by filtration using 100 mesh filter bags. The cells were discarded and the media containing the prhDNase-I was collected for additional filtration with 0.2µm filter sheets using filter-press apparatus. The DNase in the filtrated media was further purified by two steps of chromatography columns of an anion exchange resin (Poros 50HQ, Applied Biosystems, USA) followed by hydrophobic interaction chromatography of Phenyl 650C resin (Toyopearl, Japan). The purified DNase collected from the last column was 0.2 µm filtrated and stored at 4° C.

Gel electrophoresis:

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) separates proteins primarily by their molecular weight, proteins migrating as a linear function of the logarithm of their molecular weight in SDS-PAGE. For estimating the molecular weight of prh DNase I, migration on SDS-PAGE was compared to commercial molecular weight standard proteins (New England BioLabs; cat No. P7708S) and to the migration of commercially available, mammalian-cell derived recombinant human DNase I, Pulmozyme® (Genentech, CA), expressed in CHO cells. prh DNase I and Pulmozyme were analyzed by 15% Tris-Glycine SDS-PAGE.

Electrophoresis was performed using Criterion™ cell vertical electrophoresis apparatus (Bio-Rad Lab.) with premixed electrophoresis Tris-Glycine-SDS running buffer (Bio-Rad Laboratories). Fifteen percent acrylamide gels were prepared using premixed solutions of 40% Acrylamide/Bis and 10% SDS solution. Identification of the proteins was performed using two methods of detection: Coomassie brilliant blue staining and Western blot analysis using specific antibodies and Enhanced Chemical Luminescence (ECL).

Coomassie Blue Staining

Following SDS-PAGE, gels were stained with Bio-Safe™ Coomassie Stain (Bio-Rad) according to manufacturer's instructions.

Western Blotting

Western blot

Antibodies: Detection of rh DNase I was carried out by using either

(a) whole antiserum obtained from rabbits immunized against Pulmozyme®. These antibodies were prepared according to a standard polyclonal antibody preparation protocol including four immunizations with 3 mg antigen per rabbit, and collection of the serum after the fourth immunization; or

(b) affinity purified rabbit anti- prh DNase I and goat- anti- prh DNase I acquired following immunization of rabbits and goats with prh DNase and affinity purification on a Pulmozyme® column. Thus, the purified antibodies detect the common backbone sequence of Pulmozyme and prhDNase. These antibodies were prepared by GenScript USA Inc in accordance to GenScript standard polyclonal antibody preparation protocol including four immunizations with 3mg antigen per rabbit and 8 mg antigen per goat, and collection of the serum after the fourth immunization.

PAGE and Blotting: Following SDS-PAGE, proteins were transferred to a nitrocellulose membrane using iBlot™ Dry Blotting System (Invitrogen). Transfer was performed at room temperature for 8 min. Then, the membrane was blocked with 5%(v/w) non-fat dry milk powder in PBS supplemented with 0.05% (v/v) Tween-20, washed with PBS supplemented with 0.05% (v/v) Tween-20, bound to the primary antibody, followed by wash with PBS supplemented with 0.05% (v/v) Tween-20 and incubation with secondary HRP-conjugated antibody. Primary antibody was diluted 1:5000 (whole antiserum) or 1:10000 (affinity purified rabbit and goat anti prh DNase I)

in 1% (w/v) non-fat dry milk powder in PBS supplemented with 0.05% (v/v) Tween-20, and the secondary antibody-goat anti-rabbit or rabbit anti-goat HRP conjugated antibody (Jackson ImmunoResearch Laboratories, Inc., PA; cat No. 111-035-003 and cat No. 305-035-003 respectively) was diluted 1:10,000 in 1% (w/v) non-fat dry milk powder in
5 PBS supplemented with 0.05% (v/v) Tween-20. Enhanced Chemi-Luminescence (ECL) reaction was performed with ECL detection kit (Pierce; cat. No. 34080). The immunoreactive bands were detected and documented using the Molecular Imager Gel Doc XR System (Bio-Rad Laboratories, UK) for time intervals up to 60 seconds, as needed.

10 ***Isoelectric focusing***

Isoelectric Focusing (IEF) separates proteins on the basis of their charge in an electrical field. The protein migrates in a pH gradient generated by an electric field until it reaches a point in which its net charge equals zero, and that pH reflects its isoelectric point (pi). To identify the pi of rh DNase I, protein was analyzed by polyacrylamide
15 IEF gels using an XCell SureLock Electrophoresis Cell equipped with a Powerpac power supply (BIO-RAD) and by Imaged capillary isoelectric focusing (iCE) using an iCE280 analyzer (Convergent Bioscience, Toronto Canada).

IEF analysis was performed using an XCell SureLock Electrophoresis Cell equipped with a Powerpac power supply (BIO-RAD). Pre-cast polyacrylamide IEF gels
20 with a pH range of 3-7 were obtained from Invitrogen (Novex®, Invitrogen, cat No. EC6645BOX/EC66452BOX); buffers were obtained from Invitrogen (anode buffer: Novex®, cat. No LC5300; cathode buffer: Novex®, cat. No. 5370; sample buffer: Novex®, cat. No. 5371); pi protein standards were obtained from SERVA (cat. No. 39212). Electrophoresis conditions: 100mV-1h, 200mV-1h, 500mV-1.5h. Bands were
25 visualized by Bio-Safe™ Coomassie Stain (Bio-Rad, cat No. 161-0787) according to the manufacturer's instructions. The pi profile of rh DNase I (6µg/lane) was estimated using the pi protein standards, and then the banding profile was compared to that of Pulmozyme®.

For imaged capillary isoelectric focusing analysis, a sample contained prh DNase
30 I, pi markers 3.59 and 5.85 (protein simple, cat No. 102222 and 102225, respectively), 0.35% methylcellulose (protein simple, cat No. 101876), and SERVALYT pH 3-7 (SERVA, cat No. 42945.01) was introduced to the capillary through an autosampler

(PrinCE) and was focused in a capillary column under high voltage. The focusing process was monitored in real-time using whole column imaging detection (WCID). The resolved charge species appear as electrophoretic peaks that are detected at a fixed wavelength of UV280 nm. pI values and peak areas for each species were determined from the resultant electropherograms.

Mass Spectrometry

Molecular Weight Analysis

Molecular weight analysis was accomplished by mass spectrometry (MS) of rh DNase I, using a matrix-assisted laser desorption ionization time-of-flight (MALDI-ToF) mass spectrometer and sinapinic acid as a matrix. The equipment was calibrated using molecular weight standards. About 2.5 micrograms of rh DNase were used for mass analysis.

Amino Acid Sequence and structural characterization

rh DNase I sequence was analysed using reverse-phase (RP-) HPLC coupled to mass spectrometry.

The protein samples were reduced using 2.8 mM DTT (60°C for 30 minutes), modified using 8.8 mM iodoacetamide in 100 mM ammonium bicarbonate (in the dark, room temperature for 30 minutes) and digested in 10% ACN and 10 mM ammonium bicarbonate with modified trypsin (Promega) or with chymotrypsin overnight at 37°C in a 1:50 enzyme-to-substrate ratio. 3% of the resulting peptides were resolved by reversed-phase liquid chromatography on a 0.075X200-mm fused silica capillaries (J&W) packed with Reprosil reversed phase material (Dr Maisch, GmbH, Germany). Peptides were eluted with a linear 60 minutes gradient of 5 to 45% followed by 15 minutes at 95% acetonitrile with 0.1% formic acid in water at flow rates of 0.25 μ E/min. On-line mass spectrometry was performed by an ion-trap mass spectrometer (Orbitrap, Thermo) in a positive mode using repetitively full MS scan followed by collision-induced dissociation (CID) of the 7 most dominant ions selected from the first MS scan. The mass spectrometry data was analyzed using the Sequest 3.31 software (J. Eng and J.Yates, University of Washington and Finnigan, San Jose), compared to the human reference sequence.

Further sequencing and structural characterisation data was obtained from trypsin digestion of prhDNase I, with or without a further digestion with PNGaseA in the presence and absence of DTT and analysis by mass spectrometry

Disulfide Bonds and Free Sulphydryl Content

5 Human DNase-1 sequence includes four cysteine residues and contains two disulfides bonds. It was expected that the structure a recombinant human DNase-1 expressed in plant cells (prhDNase I), will be similar to the authentic human protein.

The assessment of the prhDNase I thiol content was obtained by using the Ellman's method, which is a rapid and sensitive method for quantitative analysis of the
10 total free thiols in peptides and proteins. The number of free thiols found on prhDNase I also indicates the number of cysteine residues that are engaged in disulfide bonds.

Free thiols analysis of prhDNase I and Pulmozyme® -was carried out according to standard Ellman's method under denaturing conditions. The reaction was performed by mixing the protein solution in sodium phosphate buffer with 6M Guanidine
15 Hydrochloride and the active substrate DTNB [5, 5'-Dithiobis (2-nitrobenzoic acid)]. The solutions were incubated at ambient conditions for 5-15 min. During the reaction, TNB (2-nitro-5-thiobenzoic acid) was released. A reaction of 1 mole DTNB with 1 mole thiol releases 1 mole of TNB anion with an extinction coefficient of $13,700 \text{ M}^{-1} \text{ cm}^{-1}$ at 412nm. The number of thiols was calculated as direct ratio between TNB
20 concentration and prhDNase I concentration in the analyzed sample. A protein, containing free thiols was used as positive control and DNase I formulation buffer (1mM CaCl_2 , 150mM NaCl, pH 6.1-6.5) was used as blank for analysis.

Glycan structure of rh DNase

Glycan analysis of the plant-expressed human DNaseI:

25 Samples of recombinant human DNaseI protein product from the transformed cells were reduced with DTT alkylated with iodoacetamide, and separated on a 12.5% SDS-PAGE. Bands corresponding to the correct molecular weight were excised and subjected to glycan analysis consisting of trypsin digestion followed by peptide extraction and by both PNGase A and PNGase F digestion. PNGase A digestion
30 releases all the N-linked glycans and PNGase F release all glycans except those containing a 1-3 core fucose.

The free glycans were released, purified and then labeled with the fluorescent reagent anthranilamide (2AB) followed by removal of excess 2AB.

Glycans were separated using a TSK gel Amide 80 normal phase HPLC and detected using a fluorescence detector. A Dextran hydrolysate served as a ladder for calculation of glucose unit (GU) values. Glycan profile is constructed by calculating the relative peak areas from the chromatogram of the PNGase A digestion. Assignment of the glycans is established by calculation of the GU values of the peaks found in both endoglycoside digestions and based on additional various exoglycoside digestions, comparison to known databases and confirmation of results by mass spectrometric methods.

Reverse Phase-High Performance Liquid Chromatography (RP-HPLC)

RP-HPLC was used as an analysis method for the determination of purity of hr DNase I. The analysis employed a Jupiter 3 μ , C18, 300Å, 250x4.6mm column by Phenomenex. Analyses were performed on a Dionex, Ultimate 3000 HPLC system equipped with a photodiode array detector. Chromatograms were routinely recorded at 2 wavelengths, 214 and 280nm.

Elution solvents:

Solvent A: 1 Liter, 0.1% TFA/H₂O (HPLC grade, J.T.Baker, cat # 4218-03). Solvent A was prepared by adding 1.0 mL of TFA (Sigma, cat. # T6508) to 0.999 liter of water (HPLC grade, J.T. Baker, cat. # 4218-0.3).

Solvent B: 1 Liter, 0.1% TFA/Acetonitrile (HPLC grade, Sigma, cat. # 34888). Solvent B was prepared by adding 1.0 mL TFA (Sigma, cat. # T6508) to 0.999 liter of acetonitrile (HPLC grade, Sigma, cat. # 34888).

Chromatographic method:

Chromatographic separations were performed on a Jupiter 3 μ , C18, 300Å, 250x4.6mm column (Phenomenex, cat. 00G-4263-E0). Chromatograms were obtained using a linear gradient of 5%B for 3min, followed by 5%B to 43%B for 10min, followed by 43%B to 55%B for 30min, followed by 55%B to 95%B for 10min followed by 95%B for 3min followed by 95%B to 5%B for 2min, followed by 5%B for 21 min at a flow rate of 1 ml/min and at a temperature of 55°C. Each analysis sequence was commenced with a run of DNase I formulation buffer (1mM CaCl₂ · 150mM NaCl, pH 6.1-6.5) as a blank for rh DNase I.

Samples of prh DNase I (25 μ g) in DNase I formulation buffer were analyzed. 25 μ g of the rh DNase I was pipetted into a glass insert (250 μ E), which was inserted into an HPLC auto-sampler glass vial.

Results

5 ***Characterization of plant-expressed hr-DNase I:***

Yields of hr DNase I expressed in plant cells of whole tobacco plants varied with targeting to different plant organelles. Figure 2 shows the differences in yield of plant expressed human DNase I (relative to that of apoplast targeting) when phr DNase I was expressed from a DNase I construct encoding an N-terminal ER targeting signal peptide only (APO) directing the protein to the apoplast, from a DNase I construct encoding an N-terminal ER targeting signal peptide and a C-terminal vacuolar targeting signal peptide (VAC), or from a DNase I construct encoding an N-terminal ER targeting signal peptide and a C-terminal ER retention signal peptide, targeting the protein for retention in the ER (ER). Clearly, targeting to the apoplast results in the highest yield of these three expression options. Further, Figure 2 also reveals the consistent correlation between levels of immunoreactive DNase I protein and catalytic activity.

Figure 3 shows an exemplary result of the migration of prh DNase I on SDS-PAGE (lanes 6-9), compared with mammalian cell expressed recombinant human DNase I (Pulmozyme®) (lanes 1-4). The plant expressed prh DNase I consists of one major band at a molecular weight of ~30 KDa, migrating similarly that of the commercial preparation. The prh DNase I preparation did not contain any detectable contamination, indicating a high purity. An apparent slight difference in migration characteristics was observed between the prh DNase I and the commercial preparation, with the plant expressed DNase I appearing to migrate faster. Without wishing to adhere to a single hypothesis, one possible explanation for such a difference in migration characteristics could be a difference in glycosylation patterns between the plant and mammalian cell-expressed enzymes. In order to compare for consistency between expression of phrDNase I from plants and plant cell culture, 3 batches of prh DNase I extracted from whole plants and 3 batches of prh DNase I extracted from cultured cells were analyzed on SDS-PAGE, and were observed to have the same, characteristic pattern of migration on the gel.

Figure 4 shows an exemplary result of immunological cross reactivity of the prh DNase I separated on SDS-PAGE (lanes 6-9), compared with the commercial, mammalian cell expressed recombinant human DNase I (Pulmozyme) (lanes 1-4), when reacted with unfractionated rabbit anti-human DNase I immune serum. The cross-reactive plant-expressed prh DNase I protein migrated as the 30 kDa molecular weight, similar to the commercial preparation. Further, note that the plant-expressed prh DNase I preparation does not contain any other detectable DNase I-related immunoreactive species, indicating high purity of the preparation. Three batches of DNase extracted from whole plant and 3 batches of DNase extracted from cultured cells were analyzed and have shown the same migrating characteristics. In addition, the same results were obtained using affinity purified rabbit anti-prh DNase

Figures 5A-5D shows a typical pi analysis of the plant-expressed prh DNase I as compared to Pulmozyme[®]. The rh DNase I is characterized by 3 bands; two major isoforms ranged between 4.2-4.5 and a minor isoform with a slightly lower pi (Fig. 5A, lanes 2 and 3 and 5B, lane 2). This is in contrast to the commercial standard, Pulmozyme[®] (Fig. 5A, lane 1 and 5B, lane 3), which is characterized by a variety of isoforms ranging from pi 3.5 to pi 4.5. Without wishing to adhere to a single hypothesis, one possible explanation for such a difference in pi could be a difference in glycosylation patterns between the plant and mammalian cell-expressed enzymes. Three batches of DNase extracted from whole plant and 3 batches of DNase extracted from cultured cells from cultures of different transformed BY2 lines were analyzed, and demonstrated the same migration pattern upon pi analysis. pi values for each species were determined from imaged capillary isoelectric focusing electropherograms (Figs. 5C and 5D). A main peak (MP, pi 4.41) and two acidic peaks (pre-MPI and pre-MP2 with pi of 4.27 and 4.21) were observed, consistent with the pi profile observed in the IEF gels (Figs. 5A and 5B).

Figures 6A and 6B depict an analysis of the molecular weight of plant-expressed prh DNase I by MALDI-ToF, showing the entire spectrum from 20000 to 180000 m/z (6A) or an enlargement of the prh DNase I peak at about 32000 m/z (6B). The molecular weight of the prh DNase I enzyme extracted from the plant cells was approximately 32,070 Da, while the theoretical molecular weight of the prh DNase I polypeptide, based on the proteins' 261 amino acid sequence, is 29,311 Da. Without wishing to adhere to a

single hypothesis, one likely explanation for such a difference in the chromatographic characteristics is the difference in glycosylation patterns. Therefore, glycan structures may add the remaining -2760 Daltons. Three batches of prh DNase I extracted from cultured cells were analyzed and have shown similar characteristics.

5 The difference in the molecular weight of the three prh DNase I main isoforms seen in the MALDI analysis was approximately 200 Da. (Fig 6B) This may correspond to a difference in glycan structures that can be attributed to one or two residues of N-Acetylglucosamine (203 Da). The same indication can be found in the variability of glycan structures.

10 Thus, the results of the mass spectrometry analysis confirm the molecular weight determination from the SDS-PAGE analysis (see Figure 3), and both are consistent with a molecular weight of the prh DNase I comparable to the calculated molecular weight of the expressed polypeptide, and consistent with the molecular weight of mammalian cell expressed recombinant human DNase I (Pulmozyme®).

15 ***Amino acid sequence of plant-expressed rh DNase I***

Mass spectrometry analysis of the peptides generated by trypsin and chymotrypsin digestion of plant-expressed rh DNase I showed over 97.3% coverage of the 261 DNase I amino acid sequences that matched the predicted amino acid sequence based on the DNA sequence of the expression cassette (SEQ ID NO: 6). Figure 7
20 represents the composite amino acid sequence derived from the overlapping peptides, with unconfirmed amino acids in red, putative glycosylation sites bolded and underlined, and the additional N-terminal glycine marked in green. When compared to the peptide sequence of the native human enzyme (SEQ ID NO: 6, GenBank Accession No: NP_005214.2) it is apparent that the amino acid sequence of plant-expressed rh DNase I
25 is identical to that of commercial, mammalian cell expressed dornase alfa (Pulmozyme®), and in some embodiments, with an additional glycine residue at position 1 at the N terminus, derived from the ABPI signal peptide. Indeed, further HPLC and MS analysis of a trypsin digest and chymotrypsin sub-digest of the plant-expressed rh DNase I provided a full 261 amino acid sequence, confirming the accuracy
30 of the sequence derived from overlapping peptides (FIG 7) and the identity of the amino acid sequence of the plant expressed rh DNase I and that of commercial, mammalian cell expressed dornase alfa (Pulmozyme®), with the addition of a glycine residue at position

1 at the N terminus, derived from the ABPI signal peptide. Furthermore, the analysis of the trypsin digest of prhDNase I treated and not treated with PNGase A revealed the presence of glycan sites on Asp 19 and Asp 107 as supported by the glycan analysis data in Fig 8 (below). In addition, disulphide bridges were observed between Cys102 and Cys 105 and between Cys 174 and Cys2 10.

The overlapping peptides are presented in the following coverage table (Table V), in which the first amino acid of plant-expressed rh DNase I, Gly, is numbered as amino acid 33, and where amino acids 1-32 are comprised of the ABPI signal sequence (SEQ ID NO: 4), which are cleaved from the expressed polypeptide following transfer to the endoplasmic reticulum. Table Va shows peptides produced by trypsin and PNGase digestion. In this table Gly, is denoted as amino acid number 1

Table V- Peptides Identified Following Digestion with Trypsin and Chymotrypsin

Position	% Mass	SEQ ID NO:	Sequence
33 - 48	5.325060547	17	GLKIAAFNIQTFGETK
34 - 39	2.029654905	18	LKIAAF
34 - 44	3.878161246	19	LKIAAFNIQTF
34 - 48	5.150347722	20	LKIAAFNIQTFGETK
35 - 48	4.803860015	21	KIAAFNIQTFGETK
36 - 42	2.378969069	22	IAAFNIQ
36 - 44	3.139192685	23	IAAFNIQTF
36 - 45	3.313905509	24	IAAFNIQTFG
36 - 48	4.41 1379161	25	IAAFNIQTFGETK
38 - 48	3.847234909	26	AFNIQTFGETK
39 - 48	3.629578364	27	FNIQTFGETK
40 - 44	1.906776692	28	NIQTF
40 - 48	3.178963169	29	NIQTFGETK
42 - 48	2.483049813	30	QTFGETK
43 - 48	2.090680443	31	TFGETK
49 - 57	3.019451292	32	MSNATLVSY
49 - 64	5.499783699	33	MSNATLVSYIVQILSR
53 - 64	4.264530922	34	TLVSYIVQILSR
55 - 64	3.608434794	35	VSYIVQILSR
58 - 64	2.538602758	36	IVQILSR
58 - 65	3.038226108	37	IVQILSR Y
59 - 64	2.1921 15051	38	VQILSR
60 - 64	1.888571065	39	QILSR
63 - 74	4.439055196	40	SRYDIALVQEV R
65 - 70	2.124510303	41	YDIALV
65 - 71	2.516879673	42	YDIALVQ
65 - 73	3.215808036	43	YDIALVQEV

65 - 74	3.694099266	44	YDIALVQEV
65 - 75	4.046539923	45	YDIALVQEV
65 - 78	5.079638575	46	YDIALVQEV
65 - 83	6.477641206	47	YDIALVQEV
66 - 71	2.017256323	48	DIALVQ
66 - 74	3.194475917	49	DIALVQEV
66 - 83	5.978017857	50	DIALVQEV
67 - 74	2.84203526	51	IALVQEV
68 - 73	2.017256323	52	ALVQEV
68 - 74	2.495547553	53	ALVQEV
69 - 74	2.277891008	54	LVQEV
70 - 74	1.931403301	55	VQEV
72 - 83	4.019031885	56	EV
74 - 83	3.320103521	57	R
75 - 82	2.449331437	58	DS
75 - 83	2.841812291	59	DS
75 - 106	10.83964091	60	DS
76 - 83	2.489371635	61	SH
83 - 96	4.963013347	62	K
84 - 89	2.149025427	63	LL
84 - 90	2.541394797	64	LL
84 - 91	2.893835453	65	LL
84 - 92	3.111491998	66	LL
84 - 95	4.070909143	67	LL
84 - 96	4.570532493	68	LL
84 - 97	4.990478738	69	LL
84 - 98	5.490102088	70	LL
84 - 106	8.056098967	71	LL
84 - 110	9.564293519	72	LL
84 - 112	10.43796913	73	LL
85 - 99	5.447158367	74	LD
86 - 96	3.877557079	75	DN
86 - 106	7.363123553	76	DN
87 - 106	7.010682896	77	N
88 - 106	6.661257247	78	LN
90 - 106	5.965343891	79	Q
91 - 106	5.572974522	80	D
92 - 98	2.654536986	81	AP
92 - 106	5.220533865	82	AP
93 - 106	5.00287732	83	P
94 - 106	4.705509252	84	D
95 - 106	4.353068596	85	T
97 - 102	2.246976996	86	HY
97 - 104	2.890832771	87	HY
97 - 105	3.065545596	88	HY
97 - 106	3.543836826	89	HY
97 - 107	3.893262475	90	HY
97 - 110	5.052031378	91	HY

98 - 106	3.12389058	92	YVSEPLGR
99 - 104	1.971263176	93	VVSEPL
99 - 107	2.97369288	94	VVSEPLGRN
99 - 110	4.132461783	95	VVSEPLGRNSYK
111 - 115	2.22867221	96	ERYLF
113 - 120	3.286263893	97	YLFVYRPD
113 - 122	3.982177249	98	YLFVYRPDQV
113 - 123	4.248841949	99	YLFVYRPDQVS
113 - 124	4.466498494	10	YLFVYRPDQVSA
113 - 126	5.122483137	101	YLFVYRPDQVS AVD
113 - 128	5.888771 186	102	YLFVYRPDQVS AVDSY
113 - 129	6.388394536	103	YLFVYRPDQVS AVDSYY
113 - 131	7.240458542	104	YLFVYRPDQVS AVDSYYYD
113 - 132	7.592899198	105	YLFVYRPDQVSAVDSYYYDD
113 - 133	7.767612023	106	YLFVYRPDQVSAVDSYYYDDG
114 - 128	5.389147837	107	LFVYRPDQVS AVDSY
114 - 129	5.888771 186	108	LFVYRPDQVS AVDSYY
115 - 128	5.04266013	109	FVYRPDQVS AVDSY
115 - 129	5.542283479	110	FVYRPDQVS AVDSYY
116 - 123	2.9521 15698	111	VYRPDQVS
116 - 125	3.473316229	112	VYRPDQVSAV
116 - 126	3.825756886	113	VYRPDQVS AVD
116 - 128	4.592044935	114	VYRPDQVS AVDSY
116 - 129	5.091668284	115	VYRPDQVS AVDSYY
116 - 130	5.591291634	116	VYRPDQVS AVDSYYY
116 - 131	5.94373229	117	VYRPDQVS AVDSYYYD
116 - 132	6.296172947	118	VYRPDQVS AVDSYYYDD
116 - 133	6.470885771	119	VYRPDQVS AVDSYYYDDG
118 - 128	3.788877599	120	RPDQVS AVDSY
118 - 129	4.288500948	121	RPDQVS AVDSYY
118 - 130	4.788124298	122	RPDQVS AVDSYYY
118 - 132	5.49300561 1	123	RPDQVS AVDSYYYDD
140 - 150	4.037383416	124	DTFNREPAIVR
141 - 150	3.684942759	125	TFNREPAIVR
143 - 150	2.924719144	126	NREPAIVR
143 - 151	3.375334339	127	NREPAIVRF
144 - 150	2.575293495	128	REPAIVR
153 - 159	2.74066949	129	SRFTEVR
155 - 161	2.841713132	130	FTEVREF
156 - 161	2.391097937	131	TEVREF
157 - 161	2.081489517	132	EVREF
160 - 166	2.415813937	133	EFAIVPL
160 - 172	4.095594822	134	EFAIVPLHAAPGD
160 - 175	4.834451899	135	EFAIVPLHAAPGDAVA
160 - 178	5.928764639	136	EFAIVPLHAAPGD AVAEID
160 - 180	6.492908892	137	EFAIVPLHAAPGD AVAEIDAL
160 - 181	6.992532241	138	EFAIVPLHAAPGD AVAEIDALY
160 - 182	7.344972898	139	EFAIVPLHAAPGD AVAEIDALYD

160 - 183	7.648516884	140	EFAIVPLHAAPGDAVAEIDALYDV
160 - 184	8.148140234	141	EFAIVPLHAAPGDAVAEIDALYDVY
160 - 190	10.33084718	142	EFAIVPLHAAPGDAVAEIDALYDVYLDVQEK
162 - 178	5.082765067	143	AIVPLHAAPGDAVAEID
162 - 180	5.64690932	144	AIVPLHAAPGDAVAEIDAL
162 - 181	6.146532669	145	AIVPLHAAPGDAVAEIDALY
162 - 184	7.302140662	146	AIVPLHAAPGDAVAEIDAL YDVY
163 - 181	5.928876124	147	IVPLHAAPGDAVAEIDALY
163 - 190	9.267191067	148	IVPLHAAPGDAVAEIDALYDVYLDVQEK
166 - 181	4.981476363	149	LHAAPGDAVAEIDALY
167 - 180	4.135365306	150	HAAPGDAVAEIDAL
167 - 181	4.634988656	151	HAAPGDAVAEIDALY
167 - 182	4.987429312	152	HAAPGDAVAEIDAL YD
167 - 183	5.290973298	153	HAAPGDAVAEIDALYDV
167 - 184	5.790596648	154	HAAPGDAVAEIDALYDVY
167 - 190	7.973303599	155	HAAPGDAVAEIDALYDVYLDVQEK
173 - 181	2.955207771	156	AVAEIDALY
173 - 190	6.293522714	157	AVAEIDALYDVYLDVQEK
179 - 190	4.460352897	158	ALYDVYLDVQEK
180 - 190	4.242696351	159	LYDVYLDVQEK
181 - 190	3.896208644	160	YDVYLDVQEK
182 - 190	3.396585295	161	DVYLDVQEK
182 - 191	3.966729241	162	DVYLDVQEKW
184 - 190	2.740600652	163	YLDVQEK
185 - 190	2.240977303	164	LDVQEK
185 - 191	2.81 1121249	165	LDVQEKW
185 - 199	5.533190275	166	LDVQEKWGLEDVMLM
186 - 190	1.894489595	167	DVQEK
186 - 191	2.464633542	168	DVQEKW
191 - 195	1.897439863	169	WGLED
191 - 197	2.602489733	170	WGLEDVM
191 - 198	2.94897744	171	WGLEDVML
191 - 199	3.350483324	172	WGLEDVMLM
191 - 200	3.525196149	173	WGLEDVMLMG
191 - 208	6.15195351 1	174	WGLEDVMLMGDFNAGCSY
191 - 210	6.933788727	175	WGLEDVMLMGDFNAGCSYVR
191 - 218	9.818443147	176	WGLEDVMLMGDFNAGCSYVRPSQWSSIR
192 - 198	2.378833494	177	GLEDVML
192 - 199	2.780339378	178	GLEDVMLM
192 - 208	5.581809564	179	GLEDVMLMGDFNAGCSY
198 - 214	5.9161 15429	180	LMGDFNAGCSYVRPSQW
199 - 208	3.261246422	181	MGDFNAGCSY
199 - 210	4.043081638	182	MGDFNAGCSYVR
199 - 214	5.569627722	183	MGDFNAGCSYVRPSQW
199 - 218	6.927736058	184	MGDFNAGCSYVRPSQWS SIR
200 - 208	2.859740538	185	GDFNAGCSY
200 - 210	3.641575754	186	GDFNAGCSYVR
200 - 214	5.168121838	187	GDFNAGCSYVRPSQW

200 - 215	5.434786537	188	GDFNAGCSYVRPSQWS
201 - 214	4.993409013	189	DFNAGCSYVRPSQW
203 - 214	4.190353162	190	NAGCSYVRPSQW
204 - 214	3.840927513	191	AGCSYVRPSQW
207 - 214	3.132939701	192	SYVRPSQW
207 - 218	4.491048037	193	SYVRPSQWSIR
208 - 214	2.866275001	194	YVRPSQW
209 - 214	2.366651651	195	VRPSQW
209 - 218	3.724759988	196	VRPSQWSSIR
211 - 218	2.942924771	197	PSQWSSIR
213 - 218	2.378892004	198	QWSIR
215 - 220	2.333010341	199	SSIRLW
219 - 224	2.158151613	200	LWTSPT
219 - 225	2.608766808	201	LWTSPTF
219 - 226	3.001136178	202	LWTSPTFQ
219 - 227	3.571280124	203	LWTSPTFQW
219 - 228	3.917767831	204	LWTSPTFQWL
219 - 231	4.914064263	205	LWTSPTFQWLIPD
219 - 234	5.750826164	206	LWTSPTFQWLIPDSAD
219 - 241	7.924230704	207	LWTSPTFQWLIPDSADTTATPTH
219 - 244	8.957129041	208	LWTSPTFQWLIPDSADTTATPTHCA
219 - 246	9.787860928	209	LWTSPTFQWLIPDSADTTATPTHCA
220 - 246	9.441373221	210	WTSPTFQWLIPDSADTTATPTHCA
221 - 226	2.084504524	211	TSPTFQ
221 - 227	2.654648471	212	TSPTFQW
221 - 228	3.001136178	213	TSPTFQWL
221 - 241	7.007599051	214	TSPTFQWLIPDSADTTATPTH
221 - 244	8.040497388	215	TSPTFQWLIPDSADTTATPTHCA
221 - 246	8.871229275	216	TSPTFQWLIPDSADTTATPTHCA
225 - 246	7.687979666	217	FQWLIPDSADTTATPTHCA
226 - 244	6.406632585	218	QWLIPDSADTTATPTHCA
226 - 246	7.237364471	219	QWLIPDSADTTATPTHCA
228 - 246	6.274851155	220	LIPDSADTTATPTHCA
229 - 244	5.097631562	221	IPDSADTTATPTHCA
229 - 246	5.928363448	222	IPDSADTTATPTHCA
232 - 246	4.932067017	223	SADTTATPTHCA
234 - 246	4.447745772	224	DTTATPTHCA
235 - 244	3.264573229	225	TTATPTHCA
236 - 246	3.785696695	226	TATPTHCA
239 - 246	2.948823309	227	PTHCA
245 - 250	2.060234463	228	DRIVVA
245 - 252	2.636453171	229	DRIVVAGM
245 - 253	2.982940878	230	DRIVVAGML
245 - 254	3.329428586	231	DRIVVAGMLL
245 - 255	3.807719815	232	DRIVVAGMLLR
247 - 253	2.152208992	233	IVVAGML
247 - 254	2.498696699	234	IVVAGMLL
247 - 255	2.976987929	235	IVVAGMLLR

247 - 256	3.151700754	236	IVVAGMLLRG
248 - 255	2.630500222	237	VVAGMLLR
249 - 255	2.326956236	238	VAGMLLR
250 - 255	2.023412249	239	AGMLLR
252 - 259	2.630500222	240	MLLRGAVV
254 - 266	4.11110757	241	LRGAVVPDSALPF
255 - 266	3.764619863	242	RGAVVPDSALPF
256 - 262	1.974201118	243	GAVVPDS
256 - 263	2.191857663	244	GAVVPDSA
256 - 264	2.53834537	245	GAVVPDSAL
256 - 265	2.835713438	246	GAVVPDSALP
256 - 268	4.086369477	247	GAVVPDSALPFNF
256 - 271	4.914051937	248	GAVVPDSALPFNFQAA
256 - 272	5.413675286	249	GAVVPDSALPFNFQAA Y
256 - 273	5.588388111	250	GAVVPDSALPFNFQAA YG
256 - 274	5.934875818	251	GAVVPDSALPFNFQAA YGL
256 - 276	6.553981174	252	GAVVPDSALPFNFQAA YGLSD
256 - 293	12.44599823	253	GAVVPDSALPFNFQAA YGLSDQLAQ AISDHYPVEV MLK
267 - 272	2.185617005	254	NFQAA Y
267 - 273	2.360329829	255	NFQAA YG
267 - 286	6.777625088	256	NFQAA YGLSDQLAQ AISDH Y
267 - 293	9.217939951	257	NFQAA YGLSDQLAQ AISDHYPVEV MLK
268 - 293	8.868514302	258	FQAA YGLSDQLAQ AISDHYPVEV MLK
269 - 293	8.417899107	259	QAA YGLSDQLAQ AISDHYPVEV MLK
270 - 293	8.025529737	260	AA YGLSDQLAQ AISDHYPVEV MLK
271 - 293	7.807873192	261	AYGLSDQLAQ AISDHYPVEV MLK
273 - 280	2.547459231	262	GLSDQLAQ
273 - 282	3.111603483	263	GLSDQLAQAI
273 - 285	4.150655085	264	GLSDQLAQ AISDH
273 - 289	5.646574866	265	GLSDQLAQ AISDHYPVE
273 - 290	5.950118852	266	GLSDQLAQ AISDHYPVEV
273 - 291	6.351624736	267	GLSDQLAQ AISDHYPVEVM
273 - 292	6.698112443	268	GLSDQLAQ AISDHYPVEV ML
273 - 293	7.090593298	269	GLSDQLAQ AISDHYPVEV MLK
275 - 293	6.569392766	270	SDQLAQ AISDHYPVEV MLK
277 - 293	5.95028741	271	QLAQ AISDHYPVEV MLK
279 - 293	5.211430333	272	AQ AISDHYPVEV MLK
281 - 293	4.601404418	273	AISDHYPVEV MLK
283 - 293	4.037260166	274	SDHYPVEV MLK
285 - 293	3.41815481	275	HYPVEV MLK
287 - 293	2.498585215	276	PVEV MLK

Table Va- Peptides Identified Following Digestion with Trypsin and PNGase

Position	SEQ ID NO:	Sequence
4 - 16	278	IAAFNIQTFGETK
17-32	279	MSNATLVSYIVQILSR
33 - 42	280	YDIALVQEV R

43 - 51		281	DSHLTAVGK
52 - 74		282	LLDNLNQDAPDTYHYVVSEPLGR
52 - 78		283	LLDNLNQD APDTYHYVVSEPLGRNS YK
81 - 112		284	YLFVYRPDQVSAVDSYYYDDGCEPCGNDTFNR
81 - 118		285	YLFVYRPDQVSAVDSYYYDDGCEPCGNDTFNREPAIVR
113 - 118		286	EPAIVR
119 - 122		287	FFSR
123 - 127		288	FTEVR
128 - 158		289	EFAIVPLHAAPGDAVAEIDALYDVYLDVQEK
215 - 223		290	IVVAGMLLR
224 - 261		291	GAVVPDS ALPFNFQ AA YGLS DQLAQAIS DHYPVEVMLK

Free Sulfhydryl Content (Ellman's Method)

Quantitative assay of the free-thiols indicated that both phr DNase I protein and Pulmozyme® lack free sulfhydryls, while the positive control detected four free sulfhydryls (thiols) as expected. These results confirm that in phr DNase I four Cysteine amino acids are bound in 2 disulfide bonds (Cys 102-Cys 105 and Cys 174-Cys210) as was suggested for DNase- 1 and Pulmozyme ®.

Glycan analysis:

Preliminary analysis was performed to characterize the various glycan structures of prhDNase I produced from BY2 cell culture. Figures 8A-8C show NP-HPLC profile of glycans derived from three separate batches of prhDNase I using PNGase A to release the glycans. Release of glycans by PNGase F indicated a low abundance of glycans without core fucose structures in prhDNase I. The glycan profile of all three batches of prhDNase I indicated a similar profile. Peaks in the profiles shown in Figure 8A correlate to the detailed structures in Figure 8B indicating the relative amounts of glycans released from the batches of prhDNase I.

The glycan analysis of recombinant human DNaseI protein product from the transformed tobacco cells revealed a glycan profile comprising two main glycan peaks consisting of at least four glycan structures (e.g. peaks 3+4 and 5). Predominant glycan structures representing a high percentage of the glycan profile (over 80%) contain mannose 3- β -(1,2) xylose- α -(1,3) fucose [Fc(3)M3X] and/or mannose 4- α -(1,2) xylose (M4X) with an additional one or two N-acetylglucosamine substitution on the outer mannose sugars (Figure 8B). The other glycans contain smaller structures missing either fucose or N-acetylglucosamine substitutions (3-6%) or hybrid structures (up to 14%).

Figure 8C shows a comparison of glycans found in prhDNase I released by PNGase A compared to glycans released from Pulmozyme® by PNGase F. The wide variations in glycosylation pattern found in Pulmozyme® are due to the abundance of bi- and tri-antennary glycans with additional sialic acid residues attached, leading to a mixture of charged and uncharged glycan moieties. Clearly, prh DNase I glycosylation is more homogeneous and does not include charged glycans.

Purity of plant-expressed rh DNase preparation:

Figures 8A- 8D represent a typical chromatogram of the plant-expressed prh DNase I, analyzed by RP-HPLC at 214nm (9A, 9B) and at 280nm (9C, 9D), after subtraction of start-of-sequence blank. Insets 9B and 9D are expanded views of the prh DNase I peak. In both chromatograms, the prh DNase I resolves as a main peak (> 93.6% purity) with a retention time of 28.3 minutes and an additional two small peaks, a pattern characteristic of all batches of the prh DNase I tested.

Thus, the prh DNase I preparation is characterized by molecular weight, pI, free sulfhydryl content, immune cross-reactivity and amino acid sequence comparable to but distinct from that of commercially available mammalian cell expressed DNase I, and consistent with the translation product of the expressed human DNase I coding sequence of SEQ ID NO: 9. Further, the plant-expressed rh DNase I is secreted into the medium of cells in suspension culture, and can be purified to a high degree of purity, containing less than 7% impurities detected by HPLC at both 214 and 280 nm.

***EXAMPLE 3: BIOLOGICAL ACTIVITY OF PLANT-EXPRESSED rh
DNASE I***

Biological activity of the plant-expressed rh DNase I was assayed using several assays, and compared to that of the mammalian-cell expressed commercially available human recombinant enzyme Pulmozyme® (Genentech, MA).

Methods:

DNA-Methyl Green assay

Deoxyribonuclease I (DNase I) is an endonuclease that cleaves DNA preferentially at phosphodiester linkages, yielding 5'-phosphate-terminated polynucleotides with a free hydroxyl group on position 3', on average producing tetranucleotides.

Activity of DNase I was assessed by the Methyl Green enzymatic activity assay, employing DNA from Salmon Testis (Sigma cat No. D1626) complexed with the dye methyl green (Sigma cat No. M8884) as a substrate. Methyl green intercalates between the stacked bases of double-stranded DNA. Once the long DNA molecules are hydrolyzed into tetranucleotides as a result of DNase I activity, dissociation of methyl green from the DNA occurs, the free methyl green decolorizing in a second, nonenzymatic reaction. The spontaneous decolorization of free methyl green at neutral pH is likely to result from tautomerization of the dye.

Standard curves were prepared by dilution of purified standard phrDNase I in activity Buffer (25mM HEPES-NaOH, 4mM CaCl₂, 4mM MgCl₂, 0.1% BSA, 0.05% Tween-20, pH 7.5) at concentrations ranging from 0.3 to 20 ng/ml at 2-fold series dilutions. Samples and controls were prepared in a similar matter. One hundred microliters of standards, controls and samples was added in duplicates to a 96-well plate (NUNC) containing 100µl of DNA-methyl green substrate and the contents were mixed thoroughly. The plates were then sealed and agitated for 30 min at room temperature, then incubated overnight at 37°C and absorbance measured at 620nm. Absorbance was plotted versus standards concentrations and the data were fit to a 4-parameter logistic model by the nonlinear regression method of Marquardt.

DNase I activity in different expression systems was expressed as percent of the activity measured in the apoplast targeted cells (100%). (see FIG. 2)

Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) was used to detect the presence of immunoreactive rh Dnase I in a sample. Two different ELISA methods were performed. The first ELISA assay (ELISA 1) was developed using antibodies acquired against Pulmozyme®. The second ELISA assay (ELISA 2) was developed using antibodies acquired against plant derived DNase I

The total content of prh DNase I was assessed by indirect sandwich ELISA assays. The antibodies used in ELISA 1 include unfractionated rabbit anti- human DNase I antiserum and chicken yolk IgY fraction. These antibodies were prepared following immunization of rabbits and chickens with commercially available, mammalian cell expressed human DNase I (Pulmozyme®). Unfractionated rabbit anti-human DNase I antiserum was prepared in accordance with a standard polyclonal

antibody preparation protocol including four immunizations with 3mg antigen per rabbit, and collection of the serum after the fourth immunization. Anti-human DNase I yolk IgY fraction was prepared in accordance to a standard polyclonal antibody preparation protocol including four immunizations with 2.5mg antigen per chicken, and
5 collection of the yolks after the fourth immunization.

The antibodies used in ELISA 2 included rabbit anti-prh DNase I and goat-anti-prh DNase I. These antibodies were prepared by immunization of rabbits and goats with prh DNase and affinity purification on a Pulmozyme® column. Thus, the purified antibodies detect/bind the common backbone sequence of Pulmozyme® and prh DNase
10 I. The antibodies were prepared by GenScript USA Inc in accordance to GenScript standard polyclonal antibody preparation protocol including four immunizations with 3mg antigen per rabbit and 8mg antigen per goat, and collection of the serum after the fourth immunization.

Microtiter 96-well plates (Costar cat No. 9018 for ELISA 1 and NUNC cat No. 442404 for ELISA 2) were incubated for overnight at 4°C with 100 µl/well of whole
15 rabbit antiserum 2500-fold diluted (ELISA 1) or µg/ml goat anti-prh DNase I antibody (ELISA 2) and diluted in carbonate-bicarbonate buffer pH 9.6 (Sigma cat No. C3041). Plates were then washed four times with wash buffer prepared according to manufacturer's instructions (KPL cat No. 50-63-00), blocked with 300 µl of Blocker Casein™ (Pierce cat No. 37528), incubated for one hour at 25°C and washed again four
20 times with wash buffer. A standard curve was prepared by dilution of standard prh DNase I in dilution buffer (1% (w/v) BSA in phosphate buffered saline) at concentrations ranging from 0.3125 to 20 ng/ml (ELISA 1) or 0.1562 to 10 ng/ml (ELISA 2) at 2-fold series dilutions. Similarly, samples and controls were diluted 100-
25 to 100,000-fold in dilution buffer. One hundred microliters of standards, controls and samples were added in duplicates to the wells of pre-coated and pre-blocked plates, and agitated for two hours at 25° C or incubated overnight at 4° C. Plates were then washed 4 times and incubated for 1.5 hours at 25° C with 100 µl/well of chicken yolk IgY fraction diluted 20,000-fold in the dilution buffer (ELISA 1) or rabbit anti- prh DNase I
30 antibody diluted 10,000-fold in the dilution buffer. Following four additional washing steps, 100 µl of donkey anti-chicken HRP conjugated antibody (Jackson ImmunoResearch Laboratories, Inc., PA; cat No. 703-035-155) (ELISA 1) or donkey

anti-rabbit HRP conjugated antibody (Jackson ImmunoResearch Laboratories, Inc., PA; cat No. 711-035-152) (ELISA 2), diluted 5000-fold in dilution buffer, was added and plates were incubated for one hour at 25° C. Next, plates were washed extensively and supplemented with 100 μ l TMB solution (Millipore; cat No. ESOOI). Upon color development reaction was stopped by addition of 100 μ l of 10% (v/v) HCl and then read at 450nm. Absorbance was plotted versus the standards concentrations and data were fit to a 4-parameter logistic model by the nonlinear regression method of Marquardt.

DNase I immunoreactivity in different expression systems was expressed as percent of the activity measured in the apoplast targeted cells (100%)(see FIG. 2).

10 ***Enzyme kinetics:***

The kinetics of an enzymatic reaction are typically studied by varying the concentration of substrate and plotting the rate of product formation as a function of substrate concentration (velocity). In the conventional case, this yields a typical hyperbolic Michaelis-Menten curve and a linear reciprocal Lineweaver-Burk plot, from which the kinetic constants of the enzyme can be calculated. For enzyme reactions exhibiting simple Michaelis-Menten kinetics, the Michaelis constant (K_M), or the substrate concentration at half maximum velocity ($V_{max}/2$) represents the dissociation constant of the enzyme-substrate (ES) complex or the affinity for substrate. Low K_M values indicate higher substrate affinity.

20 The kinetic constant (k_{cat}), often called k_{cat} , represents the number of substrate molecules converted into product per unit time at a single catalytic site when the enzyme is fully saturated with substrate. k_{cat} is usually the reaction rate limiting step and is calculated by dividing V_{max} by the enzyme's concentration. k_{cat}/K_M is often thought of as a measure of enzyme efficiency.

25 Many enzymes do not behave in a conventional way, their velocity curves rising to a maximum and then declining as the substrate concentration rises, referred to as substrate inhibition, and thought to be the result of binding of more than one substrate molecule to an active site. Substrate inhibition is considered a biologically relevant regulatory mechanism although it is often observed when using artificially high substrate concentrations, in a laboratory setting.

30 Kinetic plots for substrate inhibition represent variation of initial velocity as a function of substrate concentration or as double-reciprocal plot of the variation of initial

velocity with substrate concentration. Since the same compound acts at the same time as both substrate and inhibitor the double reciprocal plot does not yield a straight line. It may be assumed that inhibitory effect is negligible at low substrate concentration and from an asymptote to the curve in this region K_M and V_{max} can be estimated.

5 Assays of the enzyme kinetics of DNase I activity were performed using the DNaseAlert™ (custom made by IDT) fluorescence-quenched oligonucleotide probe, which has a HEX™ reporter dye (hexachlorofluorescein) on one end and a dark quencher on the other end, and which emits a detectable fluorescent signal upon nuclease degradation. The DNA sequence has been carefully optimized to react with a
10 wide variety of nucleases; it contains domains that will react with single-stranded endonucleases, certain single-stranded exonucleases, and double-stranded nucleases. The intact substrate has little or no fluorescence, but when cleaved by a DNase, the hydrolyzed substrate fluoresces pink (536 nm or UV excitation, 556 nm emission) when
15 cleaved, and can be detected visually or using a fluorometer. Plots of DNase I initial velocity versus substrate concentration display a velocity curve that rises to a maximum and then declines as the substrate concentration increases (Fig 10A). This pattern can characterize a typical substrate inhibition behavior. The double reciprocal plot allows the extraction of K_M and V_{max} values by abscissa intercept with an asymptote to the high substrate region (approaching the origin), where the inhibitory effect predominates (Fig
20 10B).

DNaseAlert™ based kinetic assay

DNaseAlert™ substrate (IDT; custom made) was diluted in Activity Buffer (25mM HEPES-NaOH, 4mM CaCl₂, 4mM MgCl₂, 0.1% BSA, 0.05% Tween-20, pH 7.5) at concentrations ranging from 1 to 40 μM. Ten microliters of substrate was
25 divided in duplicates into a black 96-well plate (Greiner cat No. 655900) and the reaction was initiated by rapid addition of 10 ul DNase I to a final concentration of 50 ng/mL. The plate was immediately incubated in a fluorometer and real-time data was collected (535 nm excitation, 565 nm emission) at 30 seconds intervals for 3 minutes. Initial velocities were extracted by using fluorescent product standard curve. Product
30 standard curve was prepared by dilution of 6-HEX (AnaSpec) in activity buffer at concentrations ranging from 7 to 250 nM at 1.67-fold series dilutions. Fluorescence units were transformed to concentration values (μM) by applying the calibration curve

formula ($y=ax+b$ when $y=FU$ and $a=nM$). A double reciprocal plot of initial velocity versus substrate concentration (at 2-15 μM) allows the extraction of K_M and V_{max} values by abscissa intercept with an asymptote to the high substrate region (Fig 10B).

Specific activity

5 Specific activity of human DNase I was evaluated using the DNaseAlert™ fluorescence-quenched oligonucleotide probe (supplied by Ambion, cat #AM1970, manufactured by IDT) that emits a fluorescent signal upon nuclease degradation. One unit (*U*) of DNase is defined as the amount of enzyme that releases 1 μmol of fluorescent product per minute at 22°C. The value of *specific activity* (U/mg) is calculated by dividing the value of *total activity* (*U*) by the amount of enzyme used in the reaction. Enzyme concentration was determined by absorbance at 280nm, based on extinction coefficient. The calculated extinction coefficient of phr DNase I is 1.45 g/L.

2nmol of DNaseAlert™ substrate (Ambion, cat #AM1970) was resuspended with 1 mL nuclease free water. Phr DNase I and Pulmozyme® samples were diluted 15 10,000-40,000-fold in activity buffer (25mM HEPES-NaOH, 4mM CaCl_2 , 4mM MgCl_2 , 0.1% BSA, 0.05% Tween-20, pH 7.5). 80 μL of activity buffer was divided in duplicates to a black 96-well plate (Greiner cat No. 655900). Ten microliters of substrate was then added and the reaction was initiated by rapid addition of 10 μL rh DNase I or Pulmozyme®. The plate was immediately incubated in a fluorometer and real-time data 20 was collected (535 nm excitation, 565 nm emission) at 30-second intervals for 3 minutes. Product standard curve was prepared by dilution of 6-HEX (AnaSpec cat # 81019) in activity buffer at concentrations ranging from 7 to 250 nM at 1.67-fold series dilutions. Fluorescence units were transformed to concentration values (μM) by applying the calibration curve formula ($y=ax+b$ when $y=FU$ and $a=nM$). 1 U is defined as the amount of enzyme that releases 1 μmol of fluorescent product per minute at 22°C. The value of specific activity (U/mg) was calculated by dividing the value of total activity (U) by the amount of enzyme used in the reaction.

Results:

30 ***Plant Expressed rh DNase I has superior enzyme kinetics compared to mammalian cell expressed human DNase I***

Figure 10A shows representative substrate inhibition kinetic plots of plant expressed rh DNase I and Pulmozyme®. Initial velocity versus substrate concentration

plots yielded a curve that is typical for a substrate inhibition pattern (see Table VI) for both enzymes. The K_M , and V_{max} values were calculated from asymptote equation plotted in a region of low substrate concentrations (2-15 μM) in double reciprocal plots (Figure 10B). k_{cat} values of prh DNase I and Pulmozyme® were calculated by dividing V_{max} with enzyme's concentration (Table VI), further indicating that prh DNase I and Pulmozyme® enzyme kinetics exhibit characteristics of the substrate inhibition kinetic model.

However, it is evident that although both enzymes comprise the same DNase I amino acid sequence, prh DNase I exhibits improved kinetic properties (greater substrate affinity and greater enzyme velocity) compared to Pulmozyme®. Without wishing to adhere to a single hypothesis, this may be due to unique post-translational modifications of the plant-expressed rh DNase I generated as a result of expression of the nucleic acid construct of the present invention in plant, rather than mammalian expression systems. Three batches of DNase extracted from cultured cells were analyzed and have shown similar characteristics. Further analysis of batches of DNase extracted from cells sampled from different lines of transformed BY2 cells at different times confirmed the reproducible, favorable kinetic characteristics of the plant expressed rh DNase I (see Table Via below), as compared to Pulmozyme®.

Table VI - Kinetic parameters of plant expressed rh DNase I vs. Pulmozyme®

DNase I	K_M (μM)	V_{max} ($\mu\text{M}/\text{min}$)	k_{cat} (sec^{-1})	k_{cat}/K_M ($\text{sec}^{-1}\mu\text{M}^{-1}$)
Plant-expressed rh DNase I	24.3	0.58	6.24	0.26
Pulmozyme®	60.9	0.44	4.76	0.08

Table VIa - Kinetic parameters of 3 plant expressed rh DNase I batches

prhDNase I line	K_M (μM)	V_{max} ($\mu\text{M}/\text{min}$)	k_{cat} (sec^{-1})	k_{cat}/K_M ($\text{sec}^{-1}\mu\text{M}^{-1}$)
1	16.77	0.37	5.58	0.33
2	12.54	0.36	5.44	0.43
2	12.07	0.35	5.29	0.44

Specific activity of plant-expressed rh DNase I

Specific activity values of plant-expressed rh DNase I and mammalian cell expressed DNase I (Pulmozyme®) are presented in Table VII. Significantly, the specific activity of plant-expressed rh DNase I, as assayed with the DNaseAlert™ fluorescence-quenched oligonucleotide probe is about 3-fold higher than that of the Pulmozyme®. This difference in plant-expressed rh DNase I and Pulmozyme® specific

activity reflects the improved kinetic properties of plant-expressed rh DNase I compared to Pulmozyme® (see Figures 10A and 10B and Table VI above).

Table VII - Specific activity of *plant-expressed rh DNase I* vs. Pulmozyme

DNase I	Specific activity (U/mg)
Plant-expressed rh DNase I	0.258 ± 0.055
Pulmozyme®	0.091 ± 0.018

5

Thus, plant-expressed prh DNase I is fully active catalytically, towards conventional DNase I substrates, exhibits significantly superior enzyme kinetics, and possesses a higher specific activity, consistent with the improved enzyme kinetics, when compared to that of the commercially available, clinical standard mammalian cell expressed recombinant DNase I.

10

EXAMPLE 4- SUPERIOR RESISTANCE OF PLANT-EXPRESSED rh DNASE I to ACTIN INHIBITION

Analysis of the composition of CF sputum reveals large quantities (3 to 14 mg/ml) of DNA and actin (0.06 to 5 mg/ml) released by necrosing neutrophils after their recruitment into the airways during response to infection. In addition to hydrolysis of deoxynucleic acid, DNase I can depolymerize filamentous actin (F-actin). Monomeric globular actin (G-actin) is a potent inhibitor (K_i 1 nM) of DNase I enzymatic activity, potentially influencing the effectiveness of inhaled DNase I in CF lungs.

15

To evaluate G-actin inhibitory effect on DNase I activity, an IC₅₀ assay (half maximal inhibitory concentration) was developed, applying Methyl Green enzymatic activity assay in the presence of elevated concentrations of human non-muscle actin (Cytoskeleton; cat No. APHL99).

20

One hundred microliters of actin/DNase-I mixture was divided in duplicates to a 96-well plate (NUNC cat No. 442404) containing 100ul of DNA-methyl green substrate. Human non-muscle actin (Cytoskeleton; cat No. APHL99) was diluted in activity buffer (25mM HEPES-NaOH, 4mM CaCl₂, 4mM MgCl₂, 0.1% BSA, 0.05% Tween-20, pH 7.5) containing 0.1mM ATP (Sigma cat No. A26209) to reach concentrations ranging from 100 to 0.1 ug/mL, at 2-fold series dilutions. Prh DNase I and Pulmozyme® (control) were diluted to reach concentration of 100 ng/mL. Each plate's content was

25

30

then mixed thoroughly, plates were read at 620nm, sealed and incubated for 4 hr at 37 °C and read again (620nm). The change in absorbance was plotted versus actin concentrations and IC₅₀ parameters were calculated by non-linear fit using GraFit software (Erithacus Software, UK).

5 ***Results: Plant expressed rh DNase I exhibits improved resistance to actin inhibition***

Figure 11 shows plots describing plant expressed rh DNase I and Pulmozyme® catalytic activity (expressed as AOD_{620nm}) in the presence of increasing concentrations of human G-actin. The plot of the change in absorbance versus actin concentration yields 10 hyperbolic curves allowing the extraction of IC₅₀ (Table VIII, below). Although both enzymes have the same amino acid sequence, the plant expressed rh DNase I displays greater resistance to inhibition by human actin than that of the commercial, mammalian cell expressed DNase (Pulmozyme®). Three batches of DNase extracted from cultured 15 cells were analyzed and have shown similar characteristics. Yet further, comparison of resistance/susceptibility to actin inhibition of the plant expressed rhDNase I sampled from different lines of transformed BY2 cells expressing the recombinant enzyme (see Table Villa below) confirmed the reproducible improved resistance to actin inhibition.

Without wishing to be limited to a single hypothesis, one explanation for this increased resistance to actin inhibition could be the result of the improved affinity of the 20 plant expressed enzyme for its DNA substrate, as compared to that of the mammalian expressed enzyme, or actual reduced affinity of the prhDNase I for actin monomers, or both.

**Table VIII -
IC₅₀ of plant expressed rh DNase I vs. Pulmozyme®**

DNase I	IC ₅₀ (µg/ml)
Prh DNase I	1.8191 ± 0.1003
Pulmozyme®	0.6870 ± 0.0204

25

**Table Villa -
IC₅₀ of plant expressed rh DNase I vs. Pulmozyme®**

DNase I	IC ₅₀ (µg/ml)
Prh DNase I batch 1	1.7067 ± 0.0568
Prh DNase I batch 2	1.8453 ± 0.0429
Pulmozyme®	0.5458 ± 0.0115

**EXAMPLE 5- EFFECT OF rh DNASE I ON THE RHEOLOGICAL
PROPERTIES OF SPUTUM**

Mucus is defined as the heterogeneous, adhesive, viscoelastic gel produced by goblet cells and submucosal glands. At the chemical level, mucus is an integrated structure of biopolymers. Its physical behavior is complex (non-Newtonian), with highly variable properties that are between those of a viscous liquid and an elastic solid. Characterization of the physical properties of mucus largely focuses on two properties: (i) viscous modulus, also termed loss modulus (G'' , expressed in pascal-seconds-Pa.s), which is the extent to which the gel resists the tendency to flow, and (ii) elastic modulus, also termed storage modulus (G' , expressed in pascals-Pa seconds-Pa.s), which expresses the tendency for the gel to recover its original shape following stress-induced deformation. Together, these properties describe the rheology of complex biological fluids. The phase angle or loss tangent value (δ), calculated from the inverse tangent of G''/G' ($\tan \delta = G''/G'$) is also a common parameter for characterizing mucus, reflecting the overall elastic or viscous nature of the sample and used to quantify the extent of elastic behaviour of material. A phase angle close to zero indicates strongly elastic behaviour, as opposed to 90° , which indicates purely viscous behaviour. A phase angle of 45° is considered the "cross over" value between G'' and G' .

CF Sputum collection, storage and sample treatment

CF sputum samples were collected from patients with severe lung disease attending Cystic Fibrosis Centers. Sputum was directly expectorated into a sterile hermetically sealed container and transported on ice to laboratories equipped for rheological characterization. Saliva was removed and each sputum sample was homogenized gently and divided into 200-300 mg aliquots and stored at -70°C until analyzed. Frozen samples were thawed at room temperature before analysis, as freezing of the sputum samples followed by a single thawing step was shown to afford accurate and reproducible analysis of sputum rheology, similar to those of the fresh sample before freezing.

In order to ensure that the sputum is free of exogenous DNase I activity (e.g. Pulmozyme®), sputum samples were collected 12-24 hours after treatment with Pulmozyme® aerosol, as it has been reported that inhaled aerosol DNase I is cleared from the sputum from patients as soon as two hours.

Study design

Each sputum aliquot was incubated for 30 min at 37°C with DNase I formulation buffer (1mM CaCl₂, 150mM NaCl, pH 6.1-6.5) containing either prh DNase I or Pulmozyme® (final concentration 0.2, 2, 5, , 10 and 20 ug/gr sputum).

5 Control samples were treated with DNase I formulation buffer only.

Four percent (vol/wt) of drug or control were added to the sputum sample. Following incubation, rheological properties of sputum samples were immediately measured.

Sputum rheology assay

10 Rheological properties of the sputum samples were determined using a controlled stress rheometer (HAAKE RheoStress 1, Thermo Fisher Scientific GmbH, Karlsruhe, Germany). The measurements were conducted at 20 °C using two techniques: a time-sweep (Figs. 12A-12D and 13A-13D) and a stress sweep (Figs 18A-18D and 19A-19D).

15 Time sweep measurements were performed using a 35 mm cone plate set-up. The angle between the cone and the plate was 0.5° and the sample volume required was 150 ul. Nondestructive oscillatory stress was applied to the sample and the elastic (G') and viscous (G'') modulus were recorded versus time. To avoid disruption of the weak biopolymer network in the sputum due to the oscillation forces, the measurements were performed in the linear viscoelastic region at a constant frequency of 1 Hz with a stress of 0.10 Pascals (Pa).

20 Stress sweep measurements were performed using 20mm sandblasted parallel plate geometry. The sputum samples (200 ul) were loaded onto the rheometer with a gap width of 0.5mm. A stress sweep was performed from 0.1 to 100 Pa at a constant frequency of 1 Hz and the elastic modulus, (G'), viscous modulus (G'') and phase angle (δ) were measured. The applied stress in which G' and G'' cross over (or phase angle reaches 45°) is the stress in which the sample begins to act more liquid-like than solid-like. At this point stress values were recorded and compared between DNase I treated and untreated samples.

30 Rheological parameters were determined using RheoWin 4 (HAAKE, Thermo Fisher Scientific GmbH, Karlsruhe, Germany). Before measurements, sputum samples were loaded into the rheometer plate and equilibrated for 30 seconds to allow relaxation

to the original gel structure. In order to slow down the dehydration of the sputum, a solvent trap was used. Experiments were performed on at least two sputum fractions taken from each sputum sample and the data were averaged.

5 ***Measurements of total DNA content in sputum***

Sputum DNA content was determined by the modified aminobenzoic acid (DABA) assay. Salmon sperm DNA (Sigma #D1626, ~2mg/mL) and sputum samples (-100mg) were diluted 10-fold in a dilution buffer (25mM HEPES-NaOH, 4mM CaCl₂, 4mM MgCl₂, 0.1% BSA, 0.05% Tween-20, pH 7.5) and incubated at 60°C for 1 hour. Samples were repeatedly vortexed to allow sputum disintegration. DNA concentration of the diluted salmon sperm sample was then measured (NanoDrop 2000, Thermo Fisher Scientific) and standard curve was prepared by dilution of the salmon sperm sample in the dilution buffer at concentrations ranging from 3.13 to 200 µg/ml at 2-fold series dilutions. Similarly, sputum samples were further diluted at 2-fold series dilution up to 1280-fold. Next, fifty microlitres of standards and samples was added in duplicates to a black 96-well plate (Greiner cat No. 655900) and incubated with 50 µE of 3, 5-diaminobenzoic acid (TCI Europe cat No. D0079) at 60°C for 1 hour. Fifty (50) ul of 5N HCl was then added to stop the reaction and fluorescence was measured by fluorometer (390 nm excitation /530 nm emission). Fluorescence units were plotted versus standard DNA concentrations and the data were fit to a 4-parameter logistic model by the nonlinear regression method of Marquardt. DNA concentration in sputum was then interpolated.

15 ***Magnesium chloride and rh DNase I effect on rheological properties of sputum***

25 Magnesium ion is a cofactor of DNase I, hence promoting DNA hydrolysis. Studies suggest that increasing magnesium concentration in the airway surface liquid by aerosolisation of magnesium solutions or oral magnesium supplements could improve the removal of highly viscous mucus in chronic lung disease by improving DNase activity. Furthermore, it was suggested that magnesium indirectly triggers the activity of rhDNase I in CF sputum by promoting polymerization of G-actin into F-actin, hence, reducing inhibition of DNase-I by G-actin. Consequently, magnesium supplement may

30

overcome poor response to rhDNase treatment in non-responding CF patients exhibiting low levels of magnesium in their sputum.

Magnesium sulfate and rh DNase I effect on catalytic activity of rh DNase I

Several trials conducted with inhaled magnesium sulfate in patients with acute
5 asthma have shown that magnesium sulfate inhalation is tolerable. Magnesium ions,
required for DNase I activity, can therefore be supplemented to rh DNase I formulation
as magnesium sulfate. To verify that magnesium sulfate does not produce an inhibitory
effect on rh DNase I activity a methyl green activity assay was applied in the presence of
elevated concentrations of magnesium sulfate and compared to magnesium chloride
10 effect.

One hundred microliters of plant expressed rh DNase I was divided in duplicates
to a 96-well plate (NUNC cat No. 442404) containing 100ul of DNA-methyl green
substrate to reach concentration of 100 ng/mL. Reaction was prepared in a modified
activity buffer (25mM HEPES-NaOH, 4mM CaCl₂, 0.1% BSA, 0.05% Tween-20, pH
15 7.5) containing elevated concentration of magnesium sulfate or magnesium chloride
(0.5-100mM). The plate's contents were then mixed thoroughly, and the plates were
read at 620nm, sealed and incubated for 3 hr at 37°C and re-read (620nm). Change in
absorbance was plotted versus concentrations of magnesium sulfate or magnesium
chloride added.

20 ***Results:***

Plant expressed rh DNase I is highly effective in reducing rheological properties of sputum of CF patients.

The rheological properties of sputum samples were measured following
incubation of CF sputum with DNase I formulation buffer or different concentrations of
25 plant expressed rh DNase I and Pulmozyme®, diluted in DNase I formulation buffer
(final concentration 0.2, 2, 5, 10 and 20 ug/gr sputum) (see Figures 12A- 12D ; 13A-
13D; 18A-18D and 19A-19D). DNase I concentrations correspond to the concentrations
detected 15 min after aerosolization of Pulmozyme® recommended therapeutic dose
(mean value of 2.9 µg/ml mucus).

30 Time sweep measurement (Figures 12A-12D and 13A-13D) clearly revealed that
incubation with the plant expressed rh DNase I significantly reduced sputum elastic
modulus (Figure 12A-12D) and viscous modulus (Figure 13A-13D), in a concentration-

dependent manner, and with a consistently greater efficiency (per ug DNase I; Figure 12A) than the clinically approved mammalian cell-expressed Pulmozyme®. Even greater expression of the improved rheological properties of the prh DNase I-treated sputa was observed when the reduction elastic and viscous modulus following incubation with prh DNase I was expressed as percent change compared to the untreated control (see Figures 14A and 14B). In addition, stress sweep measurements (see Figures 18A-18D and 19A-19D) reveals that the plant expressed rh DNase I disrupts the sputa elastic structure (the internal network of the sputa) in a dose- dependent manner and with greater efficiency than the clinically approved mammalian cell-expressed Pulmozyme®. The improved efficacy of the prh DNase I is consistent with the improved enzyme kinetics and the higher specific activity of the prh DNase I, compared to Pulmozyme® (see Examples 2 and 3 above).

Synergistic reduction of CF sputum rheological properties with plant expressed rh DNase I and magnesium

Time sweep measurements evaluating the rheological properties of CF sputum were performed following incubation of sputum samples with plant expressed rh DNase I protein, diluted in DNase I formulation buffer (final concentration of 2ug/gr sputum), or DNase I formulation buffer (control), in the presence or absence of 25, 50 and 100mM magnesium chloride, prepared in DNase I formulation buffer. Figures 15 and 16 clearly indicate that incubation of sputum with 25-100 mM magnesium chloride (grey bars), or prhDNase alone ("0" MgCl₂ in Figures 15 and 16) results in reduction in both the sputum elastic (Figure 15) and viscous (Figure 16) modulus. When the sputum is incubated with both magnesium chloride and plant expressed rh DNase I, (hatched bars, 25-100 mM MgC[^]), a significant synergistic reduction can be observed following a threshold of added Mg (in this experiment 100mM Mg) in both sputum rheological properties assayed [elastic (Figure 15) and viscous (Figure 16) modulus].

Stress sweep measurements were performed following incubation of sputum samples with plant expressed rh DNase I protein, diluted in DNase I formulation buffer, or DNase I formulation buffer (control), in the presence or absence of 100mM magnesium sulfate, prepared in DNase I formulation buffer (see FIGs. 20A-20C). Taken together, these results demonstrate that incubation of CF sputum with both magnesium sulfate (Figure 20A-20C, grey bars) and prhDNase (Figure 20A-20C, dark

bars) results in a synergistically greater and more significant disruption in sputum elastic structure than incubation with prhDNase or magnesium sulfate alone. This is particularly evident in patient sample A, figure 20A, where reduction in the stress sweep values for sputum sample by prhDNase was detected primarily when magnesium sulfate is added.

5 ***Magnesium salts do not inhibit plant expressed rh DNase I activity***

When catalytic activity of plant expressed rh DNase I was assayed in the presence of different concentrations of magnesium sulfate and magnesium chloride, a dose-dependent improvement of DNA hydrolytic activity was observed with concentrations between 0.5 to 10 mM magnesium, with no difference between activity
10 in the presence of chloride salt ($MgCl^+$) or sulphate salt ($MgSO_4$) (Figure 16). DNA hydrolytic activity of the plant expressed rh DNase I remained optimal with both magnesium sulfate and magnesium chloride at concentration of 10-50 mM, with only a slight depression of activity with magnesium sulphate, compared to magnesium chloride, at 100mM. Thus, neither the chloride salt ($MgCl^+$) or sulphate salt ($MgSO_4$)
15 significantly impairs DNA hydrolytic activity of the plant expressed rh DNase I over a wide range of concentrations.

Taken together, these results show that incubation with plant expressed rh DNase I improves the rheological properties of CF sputum. The dose dependent reduction in sputum rheological properties achieved with prh DNase I is more dramatic than that
20 observed with mammalian cell expressed DNase I (Pulmozyme®). Yet further, it was observed that addition of magnesium chloride can improve the effect of plant expressed rh DNase I on the rheological properties of sputum, and that the DNA hydrolytic activity of plant expressed rh DNase I is enhanced by both magnesium chloride and magnesium sulphate over a wide range of concentrations. Thus, magnesium sulfate can be
25 supplemented to prh DNase I to improve the prh DNase I's mucolytic activity *in-vivo*.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope
30 of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same

extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention. To the extent that

5 section headings are used, they should not be construed as necessarily limiting.

WHAT IS CLAIMED IS:

1. An inhalable dry powder formulation comprising a human DNase I protein and particles of a physiologically acceptable pharmacologically-inert solid carrier.
2. The inhalable dry powder formulation of claim 1, wherein said human DNase I protein is a recombinant human DNase I protein.
3. The inhalable dry powder formulation of claim 2, wherein said human DNase I protein is a plant-expressed human DNase I protein.
4. The inhalable dry powder of claim 2 or 3, wherein said human DNase I protein comprises an N-terminal Glycine residue.
5. The inhalable dry powder formulation of any one of claims 1-3, wherein said human DNase I protein comprises the amino acid sequence as set forth in SEQ ID NO: 6.
6. The inhalable dry powder formulation of claim 4, wherein said human DNase I protein comprises the amino acid sequence as set forth in SEQ ID NO:5.
7. The inhalable dry powder formulation of any one of claims 1-6, wherein said human DNase I protein has at least one core xylose and at least one core a-(1,3) fucose.
8. The inhalable dry powder formulation of any one of claims 2-7, wherein said human DNase I protein has reduced susceptibility to actin inhibition of endonuclease activity as compared with that of mammalian cell expressed human recombinant DNase I.

9. The inhalable dry powder of any one of claims 1-8, wherein said carrier is selected from the group consisting of (a) at least one crystalline sugar selected from the group consisting of glucose, arabinose, maltose, saccharose, dextrose, and lactose; and (b) at least one polyalcohol selected from the group consisting of mannitol, maltitol, lactitol, and sorbitol.

10. The inhalable dry powder formulation of any one of claims 1-9, wherein said carrier is in a form of finely divided particles having a mass median diameter (MMD) in the range of 0.5 to 10 microns.

11. The inhalable dry powder formulation of claim 10, wherein said carrier is in a form of finely divided particles having a mass median diameter (MMD) in the range of 1.0 to 6.0 microns.

12. The inhalable dry powder formulation of any one of claims 1-9, wherein said carrier is in a form of coarse particles having a mass diameter of 50- 500 microns.

13. The inhalable dry powder formulation of claim 12, wherein said coarse particles have a mass diameter of 150 microns to 400 microns.

14. The inhalable dry powder formulation of any one of claims 1-9, wherein said carrier comprises a mixture of coarse particles having a mass diameter of 150 microns to 400 micron and finely divided particles having a MMD in the range of 0.5- 10 microns.

15. The inhalable dry powder formulation of any one of claims 1-14, further comprising, as an active ingredient, a magnesium salt.

16. The inhalable dry powder formulation of any one of claims 1-15, further comprising, as an active ingredient, an agent for inhibiting formation of G actin and/or enhancing formation of F actin.

17. The inhalable dry powder of claim 1, further comprising one or more additive materials selected from the group consisting of an amino acid, a water soluble surface active agent, a lubricant, and a glidant.

18. The inhalable dry powder formulation of any one of claims 1-17, wherein said human DNase I is in association with diketopiperazine.

19. The inhalable dry powder formulation of claim 18, wherein said diketopiperazine is selected from the group consisting of succinyl diketopiperazine, glutaryl diketopiperazine, maleyl diketopiperazine, and fumaryl diketopiperazine or a pharmaceutically acceptable salt thereof.

20. The inhalable dry powder formulation of any one of claims 1-19, wherein said human DNase I protein is at least 90-95% pure human DNase I protein.

21. The inhalable dry powder formulation of any one of claims 1-19, further comprising plant beta-acetylhexosaminidase enzyme protein.

22. The inhalable dry powder formulation of claim 21, wherein said plant beta-acetylhexosaminidase enzyme protein is inactivated beta-acetylhexosaminidase enzyme protein.

23. A dry powder inhaler device, comprising the inhalable dry powder formulation of any one of claims 1-22 and a means for introducing the inhalable dry powder formulation into the airways of a subject by inhalation.

24. The dry powder inhaler device of claim 23, wherein said device is a single dose or a multidose inhaler.

25. The dry powder inhaler device of claim 23, wherein said device is pre-metered or device-metered.

26. The inhalable dry powder formulation of any one of claims 1-22 for use in reducing extracellular DNA in a subject in need thereof, the method comprising administering to the subject in need thereof an effective amount of the.

27. The inhalable dry powder formulation of claim 26, wherein said subject is suffering from a disease or condition selected from the group consisting of male infertility, metastatic cancer, a viral, bacterial, fungal or protozoan infection, sepsis, atherosclerosis, diabetes, delayed type hypersensitivity and a uterine disorder.

28. The inhalable dry powder formulation of any one of claims 1-22 for use in reducing DNA in a pulmonary secretion or fluid of a subject in need thereof.

29. The inhalable dry powder formulation of any one of claims 1-22 for use in the prevention and/or treatment of a pulmonary disease or condition associated with excess DNA in a pulmonary secretion in a subject in need thereof.

30. The inhalable dry powder formulation according to claim 28 or 29, wherein said subject is suffering from a respiratory disease selected from the group consisting of acute or chronic bronchopulmonary disease, atelectasis due to tracheal or bronchial impaction, and complications of tracheostomy.

31. The inhalable dry powder formulation of claim 30, wherein said acute or chronic bronchopulmonary disease is selected from the group consisting of infectious pneumonia, bronchitis or tracheobronchitis, bronchiectasis, cystic fibrosis, asthma, chronic obstructive pulmonary disease (COPD), TB or fungal infections.

32. The inhalable dry powder formulation of any one of claims 28-31, wherein an effective amount of said dry powder formulation is a single dose of 0.1 to 25 mg DNase I, administered daily.

33. The inhalable dry powder formulation of claim 32, wherein said effective amount of said dry powder formulation is a single dose of 0.5 to 15 mg DNase I, administered daily.

34. The inhalable dry powder formulation of claim 32, wherein said effective amount of said dry powder formulation is a single dose of 1.0 to 10 mg DNase I, administered daily.

35. The inhalable dry powder formulation of claim 32, wherein said effective amount of said dry powder formulation is a single dose of 2.0 to 5 mg DNase I, administered daily.

36. The inhalable dry powder formulation of claim 35, wherein said effective amount of said dry powder formulation is a single dose of 2.0- 3.0 mg DNase I, administered daily.

37. The inhalable dry powder formulation of claim 31, wherein an effective amount of said dry powder formulation is a plurality of doses, each dose comprising 1.0-3.0 mg DNase, said doses administered at least twice, 2-3 times, 2-4 times or 2-6 times daily.

38. The inhalable dry powder formulation of claim 31, wherein an effective amount of said dry powder formulation is a plurality of doses, each dose comprising 1.0-3.0 mg DNase, said doses administered once every 36 hours, once every 36-48 hours, once every 36-72 hours, once every 2-3 days, once every 2-4 days, once every 2-5 days, or once every week.

39. The inhalable dry powder formulation of claim 31, wherein an effective amount of said dry powder formulation is a plurality of doses, each dose comprising 1.0-3.0 mg DNase, said doses administered once every 36 hours, once every 36-48 hours, once every 36-72 hours, once every 2-3 days, once every 2-4 days, once every 2-5 days, or once every week.

(a) -----MRCMKLLCALALCALAALGGVYSLKIAAFNIQTFGETKMS
 (b) MIVLSVGSASSSPIVAVVFSVALLLFYFSETSLGLKIAAFNIQTFGETKMS
 1 50

(a) NATLVSYIVQILSRVDIALVQEVDRDShLTAVGKLLDNLNQDAPDTYHYVV
 (b) NATLVSYIVQILSRVDIALVQEVDRDShLTAVGKLLDNLNQDAPDTYHYVV
 51 100

(a) SEPLGRNSYKERYLFFVYRDPQVSAVDSYYDDGCEPCGNDTFNREPAIVR
 (b) SEPLGRNSYKERYLFFVYRDPQVSAVDSYYDDGCEPCGNDTFNREPAIVR
 101 150

(a) FFSRFFTEVREFAIVPLHAAPGDVAEIDALYDVYLDVQEKWGLEDEVMLMG
 (b) FFSRFFTEVREFAIVPLHAAPGDVAEIDALYDVYLDVQEKWGLEDEVMLMG
 151 200

(a) DENAGCSYVRPSQWSSIRLWTSPTFQWLI PDSADTTATPTHCAVDRI VVA
 (b) DENAGCSYVRPSQWSSIRLWTSPTFQWLI PDSADTTATPTHCAVDRI VVA
 201 250

(a) GMLLRGAVVPDSALPFNFQAAYGLSDQLAQAI SDHYPPVEVMLK
 (b) GMLLRGAVVPDSALPFNFQAAYGLSDQLAQAI SDHYPPVEVMLK
 251 295

FIG. 1

FIG. 2

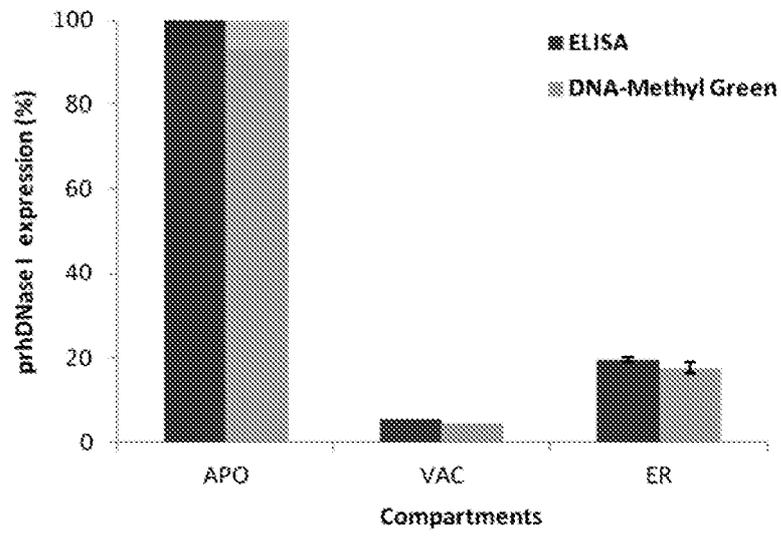


FIG. 3

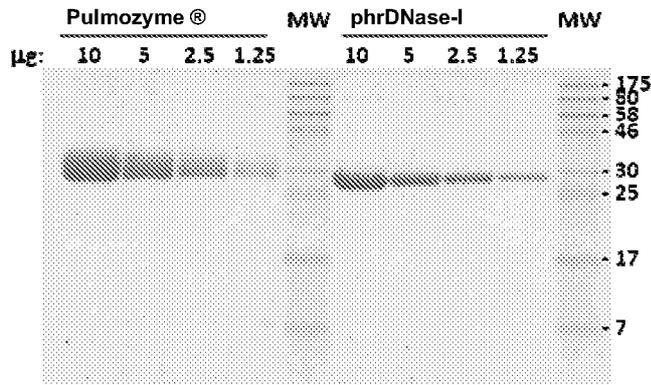


FIG. 4

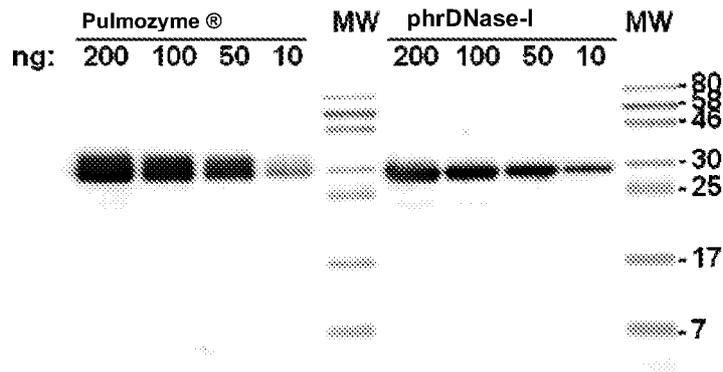


FIG. 5A

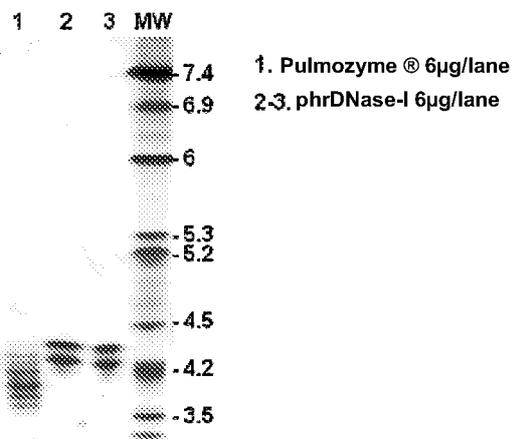


FIG. 5C

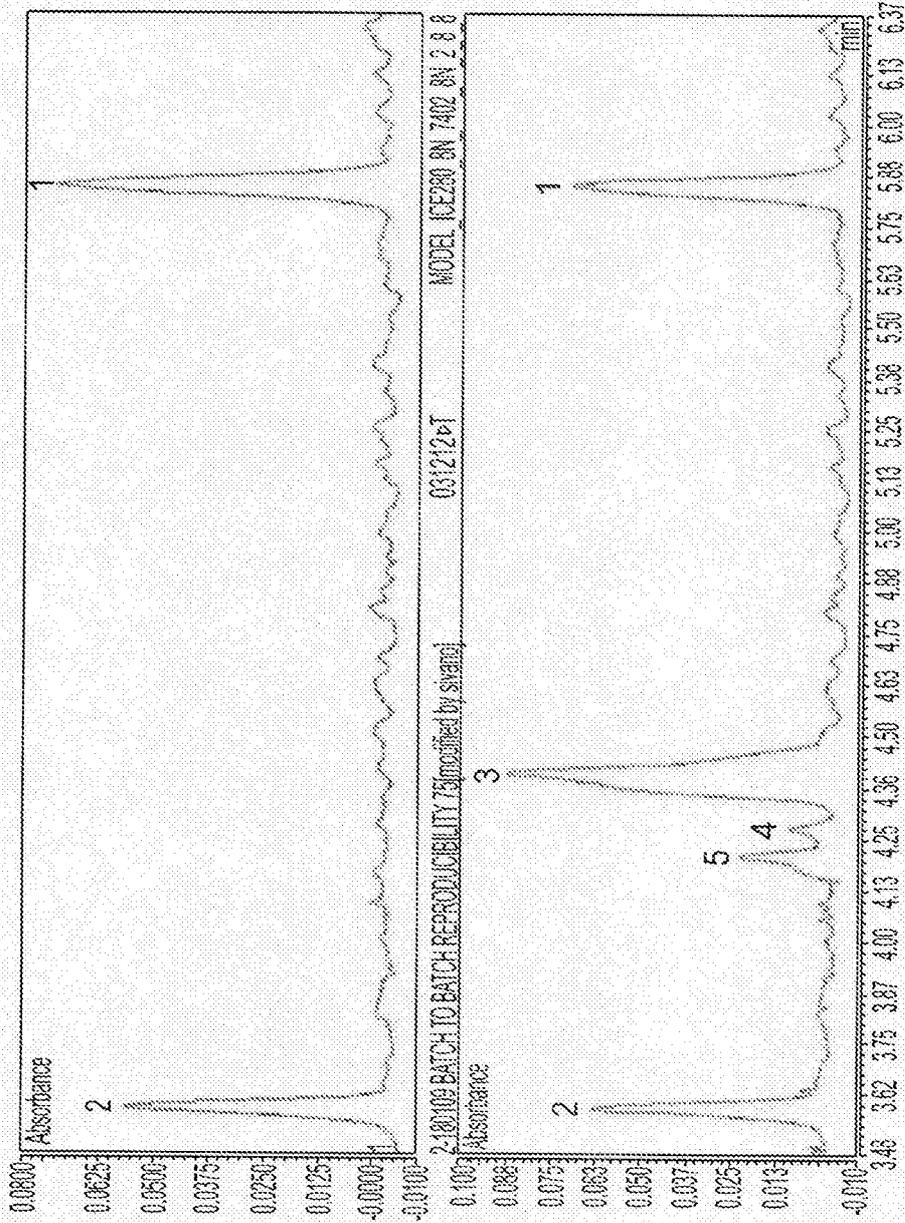


FIG. 5D

FIG. 5B

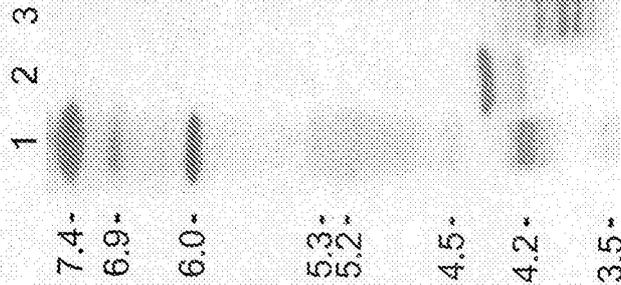


FIG. 6A

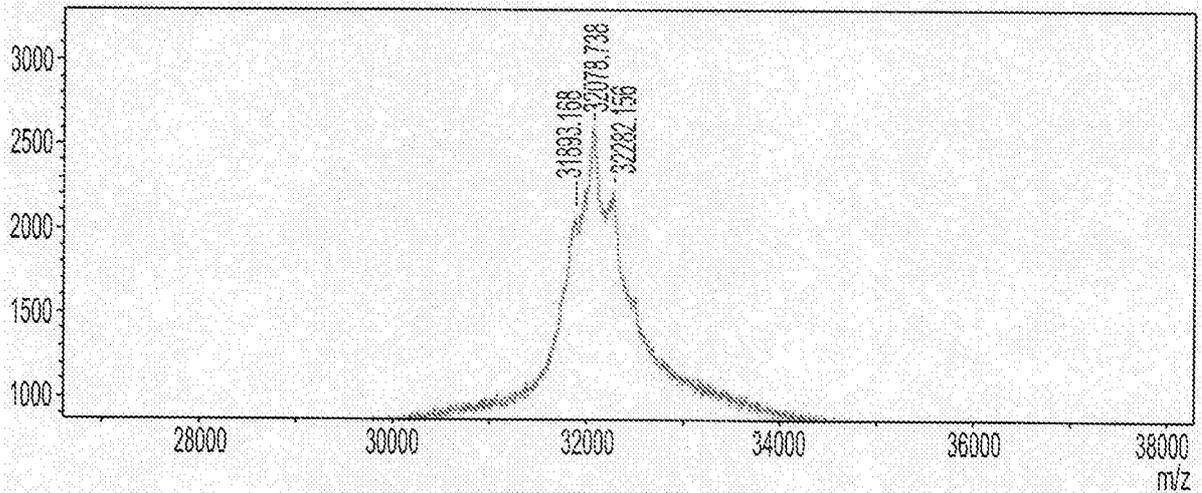
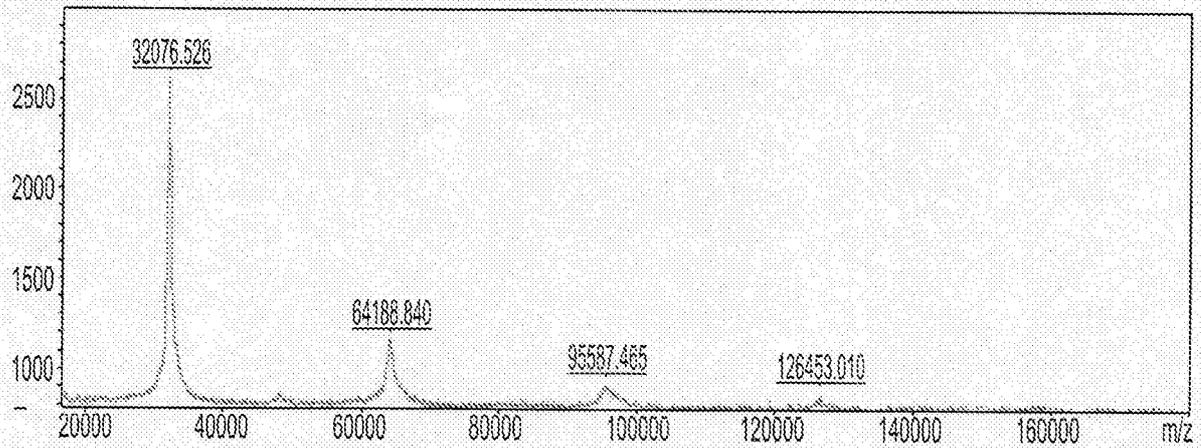


FIG. 6B

1	GLKIAAFNIQ	TFGETKMSNA	TLVSYIVQIL	SRYDIALVQE	VRDShLTAVG
51	KLLDNLNQDA	PDTYHYVVSE	PLGRNSYKER	YLFVYRPDQV	SAVDSYYYDD
101	GCEPCGNDTF	NREPAIVRFF	SRFTEVREFA	IVPLHAAPGD	AVAEIDALYD
151	VYLDVQEKWG	LEDVMLMGDF	NAGCSYVRPS	QWSSIRLWTS	PTFQWLIPDS
201	ADTTATPTHC	AYDRIVVAGM	LLRGAVVPDS	ALPFNFQAAY	GLSDQLAQAI
251	SDHYPVEVML	K			

FIG. 7

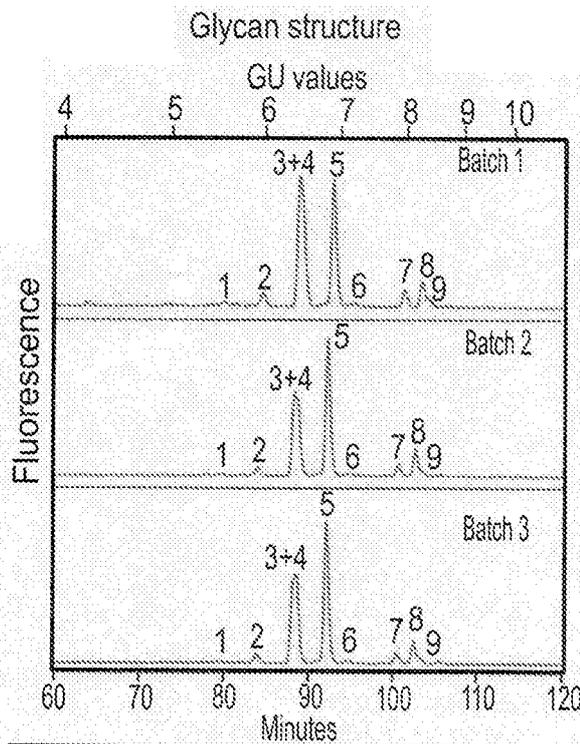


FIG. 8A

Peak Number		1	2	3+4			5	6	7	8	9		
glycan formula		A1X	M4X	FcM3X	FcA[6]1X	M4XA1	FcA[3]1X	FcA2X	hybrid				
Glycan structure									UNDETERMINED				
calculated MW		1268	1227	1211	1414	1430	1414	1618					
average GU value		5.5	5.9		6.4		6.9	7.2	8.1	8.3	8.5		
Batch #	1	%relative area	2	4	48			33	2	4	6	2	
		Detected MW	1268	ND	1211	1415	1431	1415	1618	ND	ND	ND	ND
	2	%relative area	ND	1	2	25	16		43	ND	3	8	2
		Detected MW	ND	1228	ND	1415	ND	ND	1618	ND	ND	ND	ND
	3	%relative area	ND	1	2	22	20		42	ND	4	7	3
		Detected MW	ND	1228	ND	1415	ND	1415	1617	ND	ND	ND	ND

ND-not detected

FIG. 8B

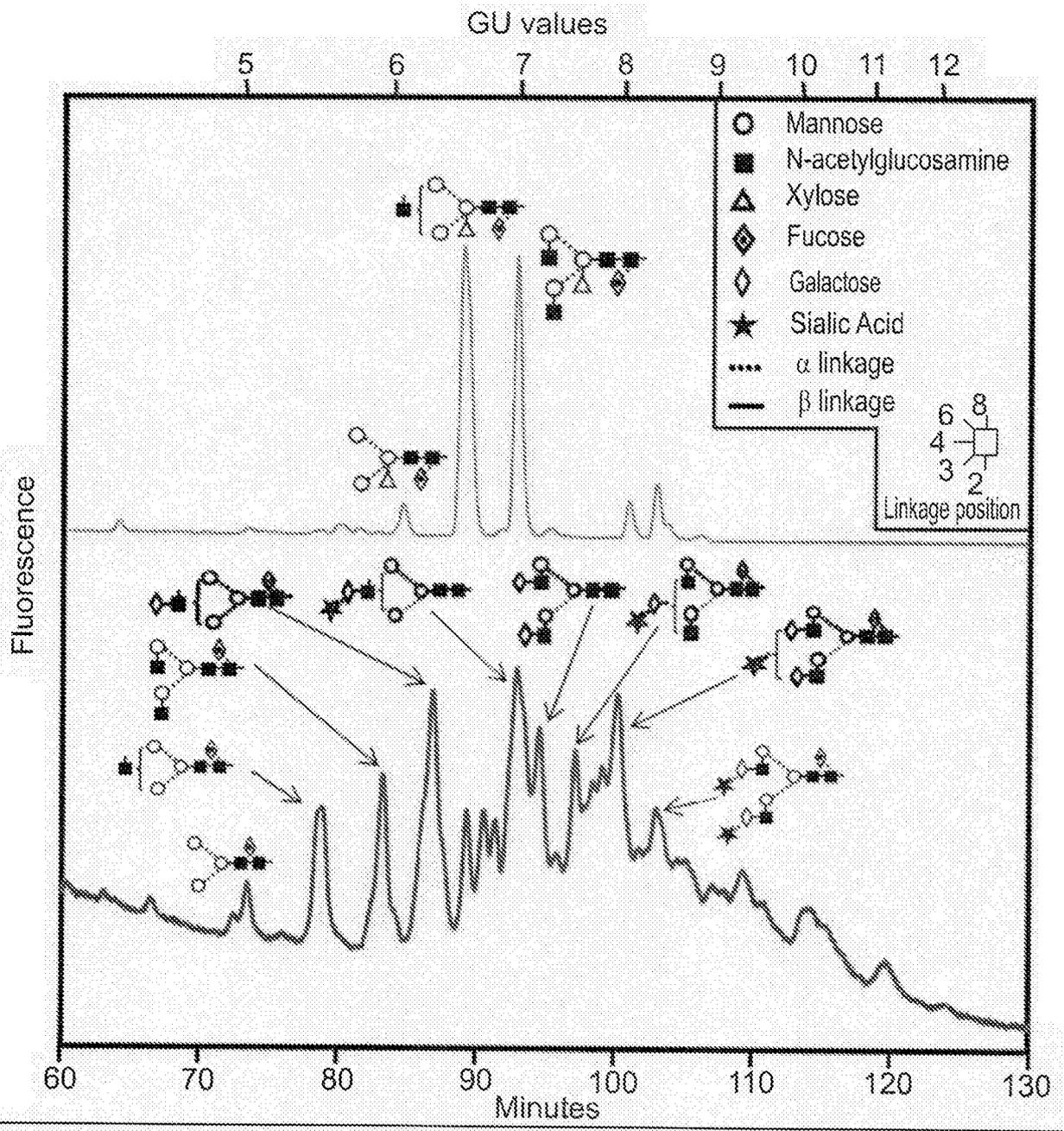


FIG. 8C

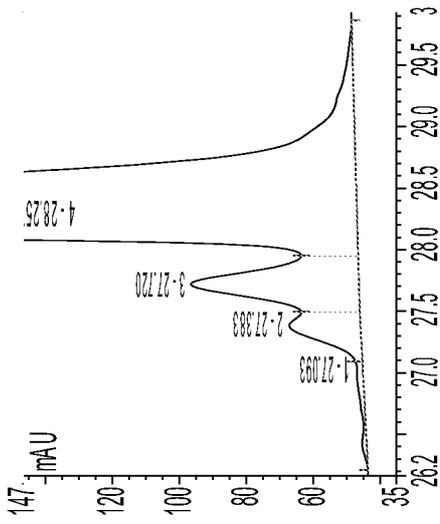


FIG. 9B

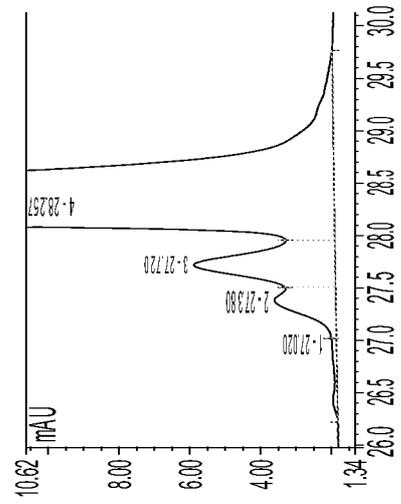


FIG. 9D

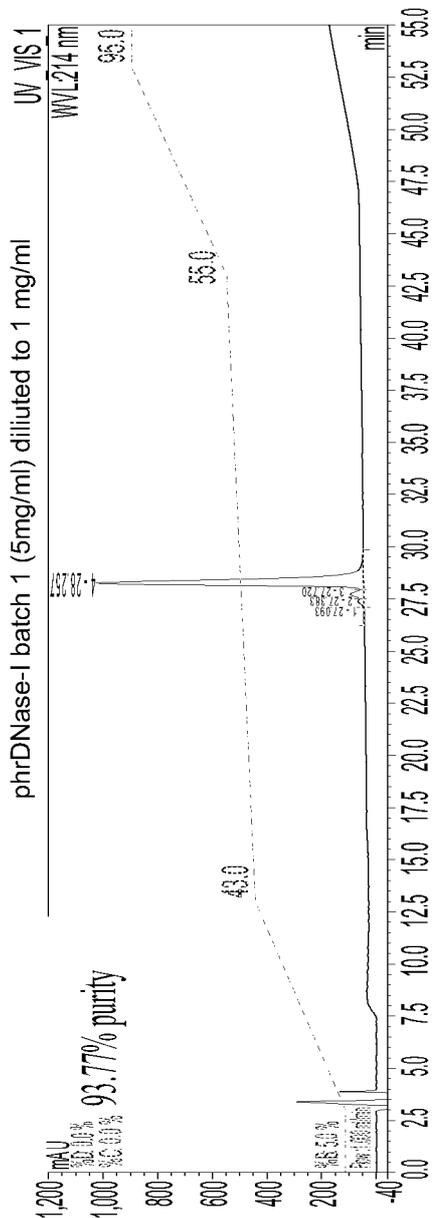


FIG. 9A

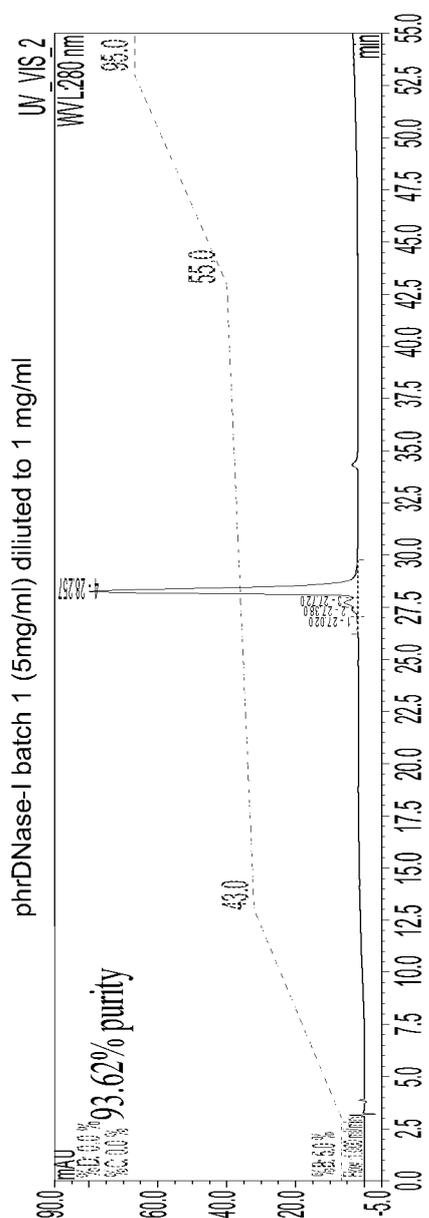


FIG. 9C

FIG. 10A

10A.

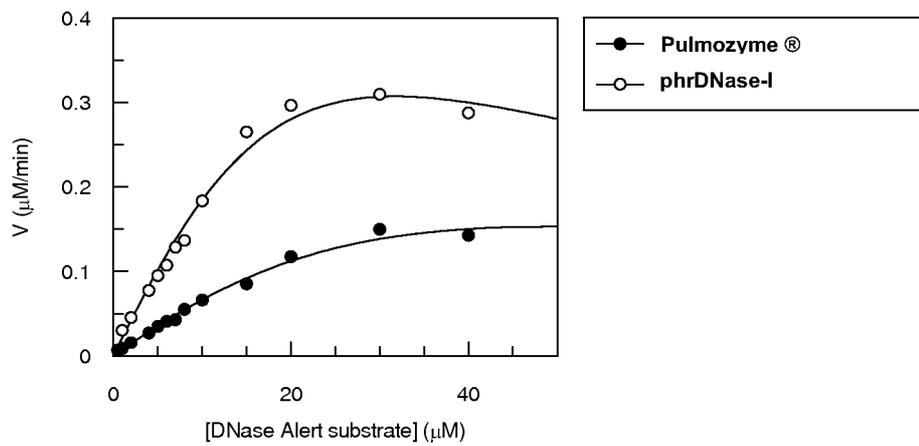


FIG. 10B

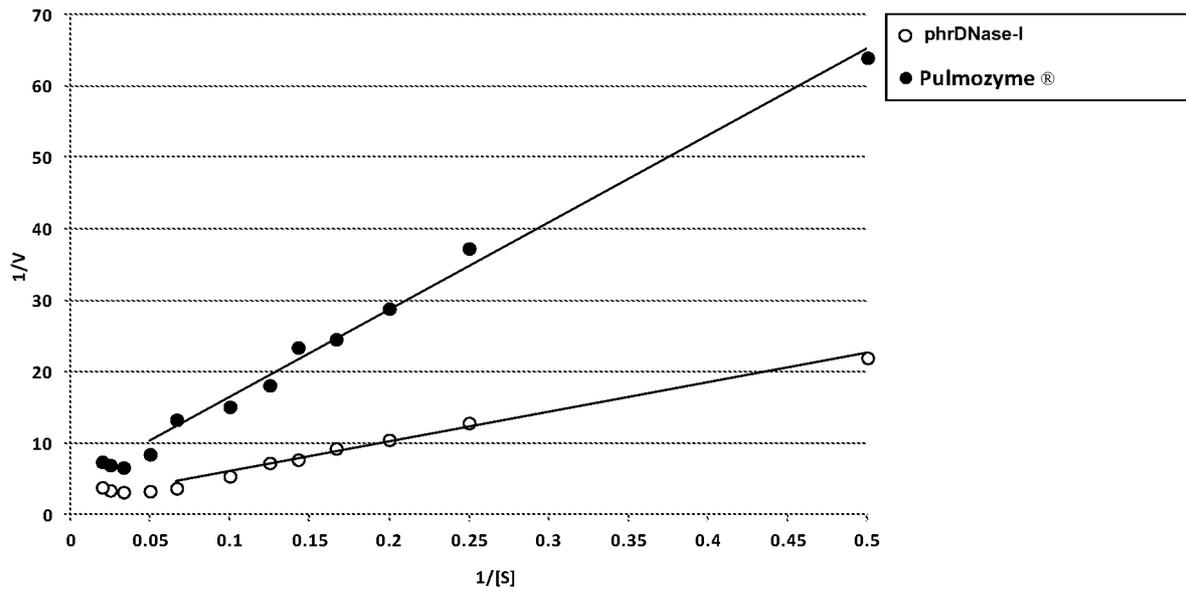
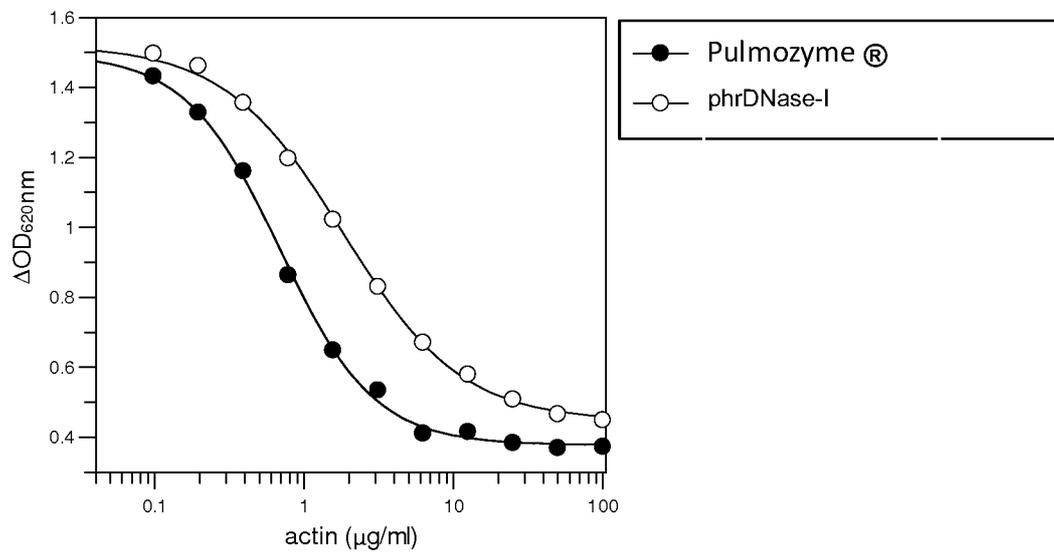


FIG. 11



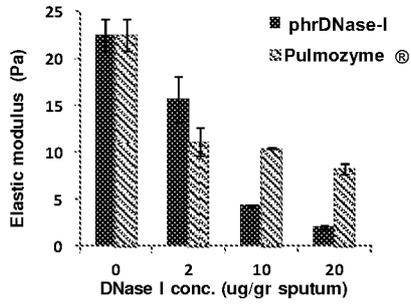


FIG. 12A

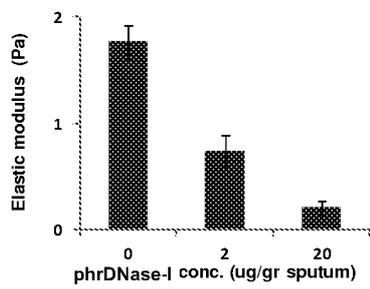


FIG. 12B

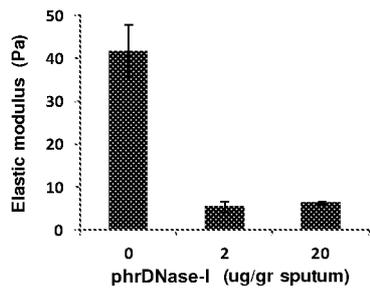


FIG. 12C

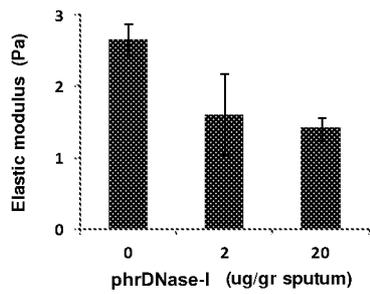


FIG. 12D

FIG. 13A

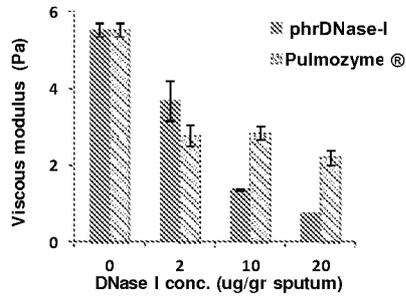


FIG. 13B

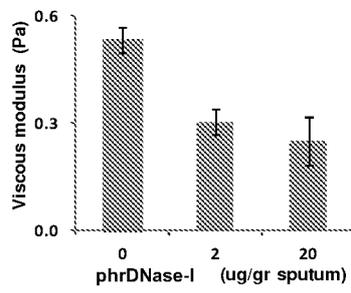


FIG. 13C

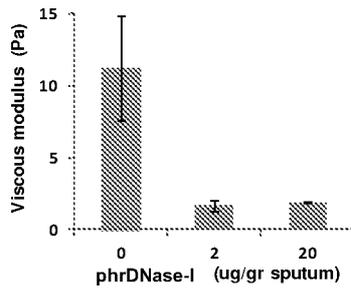


FIG. 13D

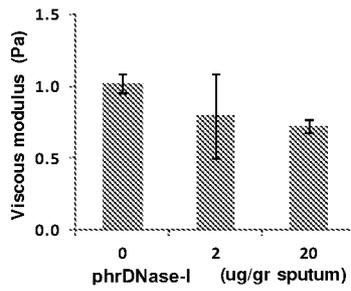


FIG. 14A

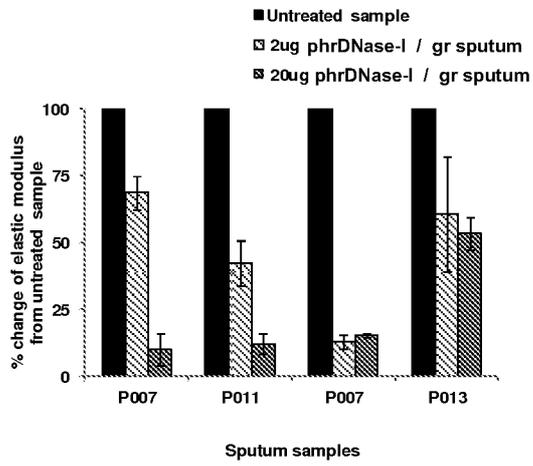


FIG. 14B

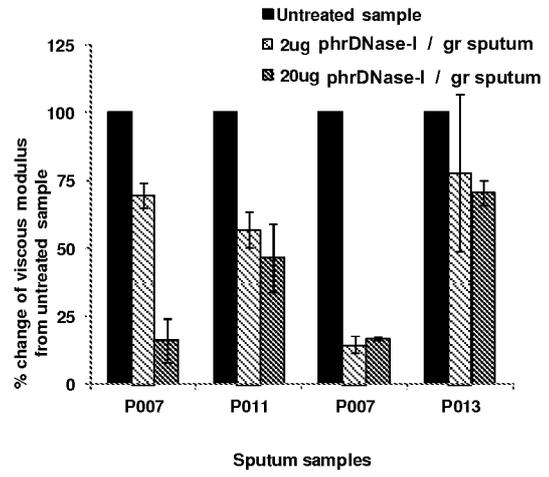


FIG. 15

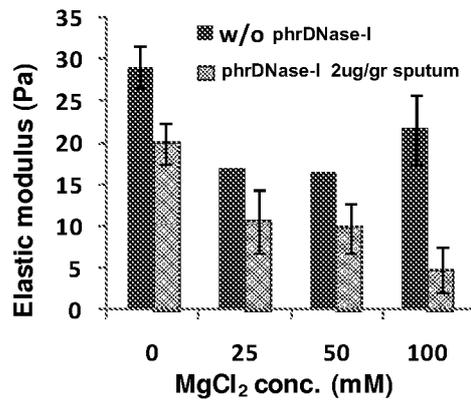


FIG. 16

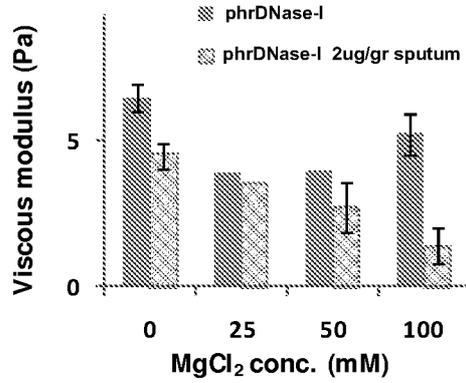


FIG. 17

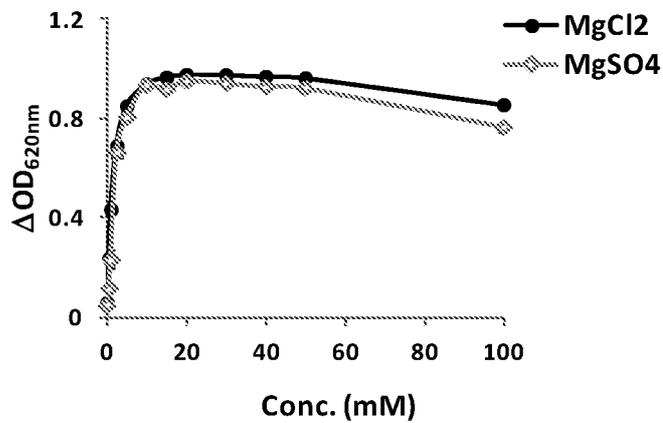


FIG. 18A

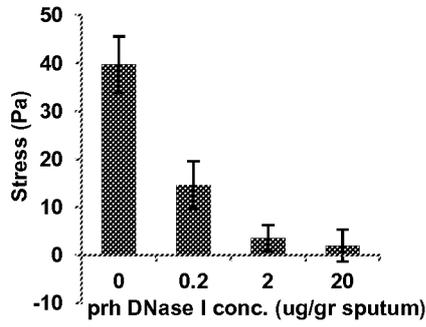


FIG. 18B

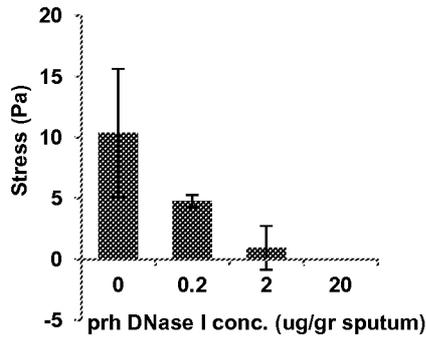


FIG. 18C

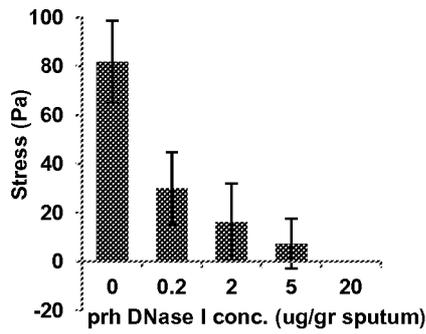


FIG. 18D

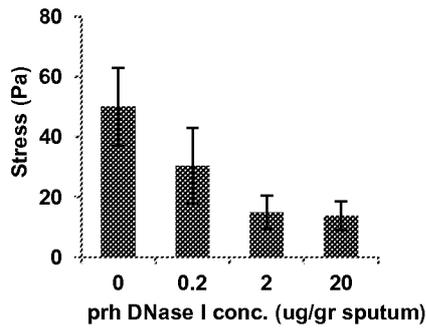


FIG. 19A

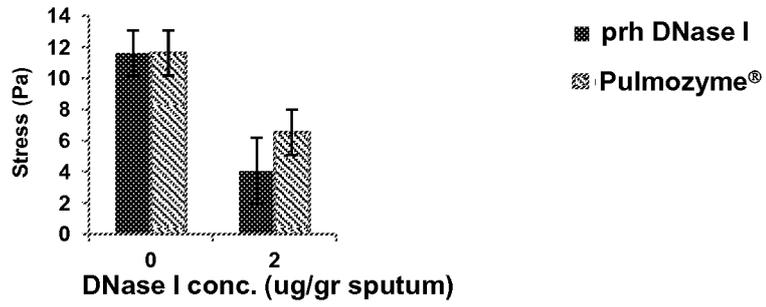


FIG. 19B

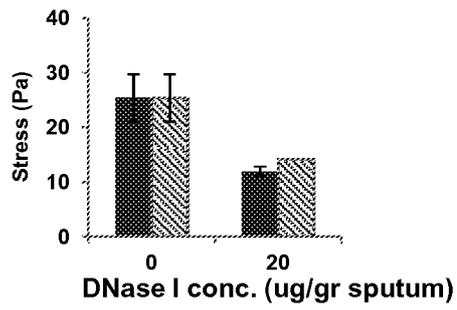


FIG. 19C

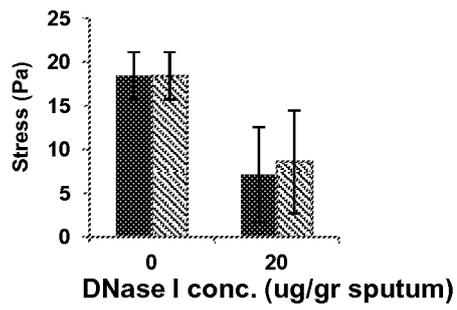
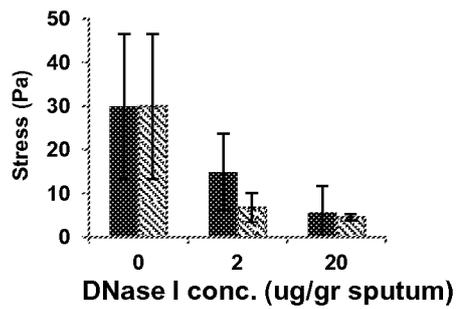


FIG. 19D



■ w/o MgSO₄
■ with 100mM MgSO₄ in formulation buffer

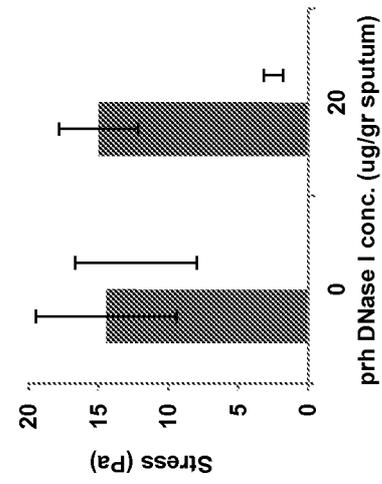


FIG. 20A

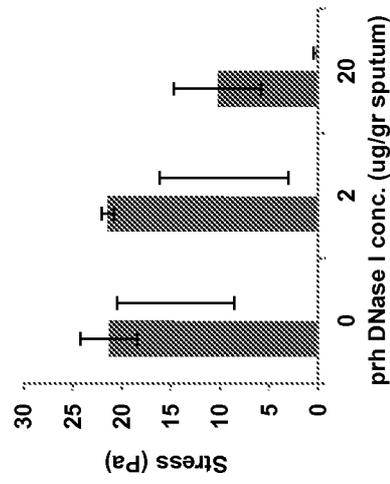


FIG. 20B

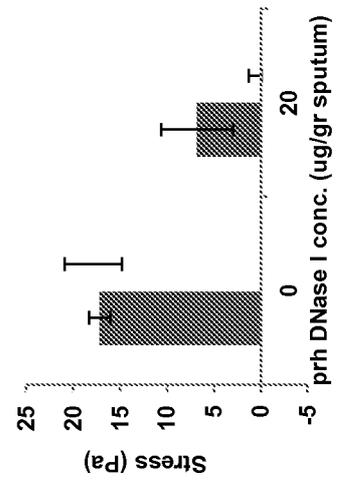


FIG. 20C

INTERNATIONAL SEARCH REPORT

International application No
PCT/IL2013/050094

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/22
 ADD.
 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)
C12N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal , WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Y,P	whole document esp. paragraph [198] -----	4,7-39
X	wo 2011/053982 A2 (UNIV WASHINGTON [US] ; LEDBETTER JEFFREY A [US] ; HAYDEN-LEDBETTER MARTH) 5 May 2011 (2011-05-05) whole document esp paragraphs [13,22,87,90,130,172,174-175,177,180,283,292,310] , claim 62, and seq id no 147 -----	1-39
Y	wo 2009/123950 A2 (UNIV PENNSYLVANIA [US] ; DI RI ENZO JOSEPH M [US]) 8 October 2009 (2009-10-08) whole document esp. paragraphs [3,4,15,181,191,194,196,216,217,224,228,232] and seq id no 4 -----	1-39
	-/- .	

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See patent family annex.

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Date of the actual completion of the international search 4 July 2013	Date of mailing of the international search report 24/07/2013
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Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Bruck, Mari anne
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INTERNATIONAL SEARCH REPORT

International application No

PCT/IL2013/050094

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	whol e document esp. pages 1, 15, cl aims 17-22 and seq i d no 1 -----	1-39
X	US 2007/196503 AI (WI LSON BRYAN R [US] ET AL) 23 August 2007 (2007-08-23)	18, 19
Y	whol e document esp. paragraphs [7,8,48,61 ,69] -----	1-39
X	wo 2005/056037 AI (MEDPHARM LTD [GB] ; BROWN MARC BARRY [GB] ; JONES STUART ALLEN [GB] ; MAR) 23 June 2005 (2005-06-23)	1,23-25
Y	whol e document esp. cl aims -----	2-39

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No PCT/IL2013/050094
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wo 2005056037	AI	23-06-2005	NONE
