

- 1 -

## FIBRINOLYTICALLY ACTIVE POLYPEPTIDE

5                    Field of the Invention

                  This invention relates to a fibrinolytically  
active metalloproteinase of non-naturally occurring  
sequence, to combinant methods for its manufacture, and  
10 to its use in treating thrombosis in vivo.

Background of the Invention

                  Fibrolase is an enzymatically active  
15 polypeptide (specifically, a metalloproteinase)  
composed of 203 amino acid residues that was originally  
isolated by purification from the venom of the Southern  
Copperhead snake; United States Patent No. 4,610,879,  
issued September 9, 1986 (Markland et al.); and Guan et  
20 al., Archives of Biochemistry and Biophysics, Volume  
289, Number 2, pages 197-207 (1991). The enzyme  
exhibits direct fibrinolytic activity with little or no  
hemorrhagic activity, and it dissolves blood clots made  
either from fibrinogen or from whole blood.

25  
                  The amino acid sequence of fibrolase has also  
been determined, with methods described for recombinant  
production in yeast and use for the treatment of  
thrombembolic conditions in vivo; Randolph et al.,  
30 Protein Science, Cambridge University Press (1992),  
pages 590-600, and European Patent Application No.  
0 323 722 (Valenzuela et al.), published July 12, 1989.

- 2 -

Summary of the Invention

This invention provides a fibrinolytic  
5 metalloproteinase having the non-naturally occurring  
linear array of amino acids depicted in SEQ ID NO: 1,  
also referred to herein as "novel acting thrombolytic"  
(or "NAT"). Also provided are nucleic acid molecules,  
such as the one of SEQ ID NO: 2 and variants thereof  
10 encoding NAT.

The term "mature" is used in its conventional  
sense to refer to the biologically active polypeptide  
which has been enzymatically processed *in situ* in the  
15 host cell to cleave it from the prepro region.

Because of its fibrinolytic activity, NAT is  
useful *in vivo* as a blood clot lysing agent to treat  
thrombosis in a mammal (including rats, pigs and  
20 humans).

The NAT polypeptide of this invention provides  
advantages over naturally occurring fibrolase as a  
therapeutic agent (i.e., the fibrinolytic polypeptide  
25 found in snake venom). Native fibrolase is known to  
contain several alternate N-termini: QQRFP, EQRFP and  
ERFP (in which "E" designates a cyclized glutamine, or  
pyroglutamic acid). More specifically, starting with  
an N-terminus composed of QQRFP, the fibrolase molecule  
30 undergoes degradation to result in two isoforms, having  
N-terminal sequences of EQRFP and ERFP, respectively.  
Recombinant fibrolase as produced in yeast typically  
yields a mixture of all three of these forms and is  
thus not homogeneous. See Loayza et al., Journal of  
35 Chromatography, B 662, pages 227-243 (1994). Moreover,

- 3 -

the cyclized glutamine residue results in a "blocked" N-terminus which makes sequencing impossible.

5 In contrast, the recombinant NAT of this invention provides a single species: only one N-terminus is typically produced. The result is greater homogeneity of the end product compared to recombinant fibrolase, which is beneficial when medical applications are the intended end use.

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#### Brief Description of the Figures

FIGURE 1 depicts in linear fashion the full amino acid sequence of NAT (SEQ ID NO: 3), consisting  
15 of the "prepro" region (underscored), which includes the "signal" peptide, and the mature polypeptide (non-underscored).

#### Detailed Description of the Invention

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NAT may be produced by recombinant expression of the nucleic acid molecule of SEQ ID NO: 4, which encodes the full amino acid sequence of NAT (SEQ ID NO: 3), including the prepro region from nucleotides 1-783  
25 and mature polypeptide from nucleotides 784-1386, in a suitable host. Following expression, the prepro region is enzymatically processed off in the host cell to yield the mature active polypeptide (SEQ ID NO: 1).

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Preferably, NAT is produced recombinantly in yeast, as will be explained in greater detail further below.

The mature polypeptide (SEQ ID NO: 1) which  
35 is thus produced may or may not have an amino terminal

- 4 -

methionine, depending on the manner in which it is prepared. Typically, an amino terminal methionine residue will be present when the polypeptide is produced recombinantly in a non-secreting bacterial  
5 (e.g., *E. coli*) strain as the host.

Besides the nucleic acid molecule of SEQ ID NO: 2, also utilizable are degenerate sequences thereof which encode the same polypeptide. The present  
10 invention also embraces nucleic acid molecules that may encode additional amino acid residues flanking the 5' or 3' portions of the region encoding the mature polypeptide, such as sequences encoding alternative pre/pro regions (i.e., sequences responsible for  
15 secretion of the polypeptide through cell membranes) in place of the "native" pre/pro region (i.e., found in naturally occurring fibrolase). The additional sequences may also be noncoding sequences, including regulatory sequences such as promoters of transcription  
20 or translation, depending on the host cell.

NAT can be prepared using well known recombinant DNA technology methods, such as those set forth in Sambrook et al., Molecular Cloning: A  
25 Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989) and/or Ausubel et al., editors, Current Protocols in Molecular Biology, Green Publishers Inc. and Wiley and Sons, New York (1994). A DNA molecule encoding the polypeptide or truncated  
30 version thereof may be obtained, for example, by screening a genomic or cDNA library, or by PCR amplification, to obtain a nucleic acid molecule encoding fibrolase, followed by replacement of the codons encoding the N-terminal amino acid residues QQR  
35 with a codon for serine (S). Alternatively, a DNA molecule encoding NAT may be prepared by chemical

- 5 -

synthesis using methods well known to the skilled artisan, such as those described by Engels et al. in Angew. Chem. Intl. Ed., Volume 28, pages 716-734 (1989). Typically, the DNA will be several hundred  
5 nucleotides in length. Nucleic acids larger than about one hundred nucleotides can be synthesized as several fragments using these same methods and the fragments can then be ligated together to form a nucleotide sequence of the desired length.

10

The DNA molecule is inserted into an appropriate expression vector for expression in a suitable host cell. The vector is selected to be functional in the particular host cell employed (i.e.,  
15 the vector is compatible with the host cell machinery, such that expression of the DNA can occur). The polypeptide may be expressed in prokaryotic, yeast, insect (baculovirus systems) or eukaryotic host cells, although yeast is preferred as will be explained in  
20 greater detail further below.

The vectors used in any of the host cells to express NAT may also contain a 5' flanking sequence (also referred to as a "promoter") and other expression  
25 regulatory elements operatively linked to the DNA to be expressed, as well as enhancer(s), an origin of replication element, a transcriptional termination element, a complete intron sequence containing a donor and acceptor splice site, a signal peptide sequence, a  
30 ribosome binding site element, a polyadenylation sequence, a polylinker region for inserting the nucleic acid encoding NAT, and a selectable marker element. Each of these elements is discussed below.

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Optionally, the vector may also contain a "tag" sequence, i.e., an oligonucleotide sequence

- 6 -

located at the 5' or 3' end of the polypeptide-coding sequence that encodes polyHis (such as hexaHis) or another small immunogenic sequence (such as the c-myc or hemagglutinin epitope, for which antibodies, including monoclonal antibodies, are commercially available). This tag will be expressed along with NAT, and can serve as an affinity tag for purification of this polypeptide from the host cell. Optionally, the tag can subsequently be removed from the purified polypeptide by various means, for example, with use of a selective peptidase.

The 5' flanking sequence may be the native 5' flanking sequence, or it may be homologous (i.e., from the same species and/or strain as the host cell), heterologous (i.e., from a species other than the host cell species or strain), hybrid (i.e., a combination of 5' flanking sequences from more than one source), or synthetic. The source of the 5' flanking sequence may be any unicellular prokaryotic or eukaryotic organism, any vertebrate or invertebrate organism, or any plant, provided that the 5' flanking sequence is functional in, and can be activated by the host cell machinery.

The origin of replication element is typically a part of prokaryotic expression vectors purchased commercially and aids in the amplification of the vector in a host cell. Amplification of the vector to a certain copy number can, in some cases, be important for optimal expression of NAT. If the vector of choice does not contain an origin of replication site, one may be chemically synthesized based on a known sequence, and then ligated into the vector.

The transcription termination element is typically located 3' to the end of the polypeptide

- 7 -

coding sequence and serves to terminate transcription of the mRNA. Usually, the transcription termination element in prokaryotic cells is a G-C rich fragment followed by a poly T sequence. While the element is easily cloned from a library or even purchased commercially as part of a vector, it can also be readily synthesized using known methods for nucleic acid synthesis.

10           A selectable marker gene element encodes a protein necessary for the survival and growth of a host cell grown in a selective culture medium. Typical selection marker genes encode proteins that: (a) confer resistance to antibiotics or other toxins, for example, 15   ampicillin, tetracycline or kanamycin for prokaryotic host cells, zeocin for yeast host cells, and neomycin for mammalian host cells; (b) complement auxotrophic deficiencies of the cell; or (c) supply critical nutrients not available from complex media. Preferred 20   selectable markers for use in prokaryotic expression are the kanamycin resistance gene, the ampicillin resistance gene, and the tetracycline resistance gene.

          The ribosome binding element, commonly called 25   the Shine-Dalgarno sequence (for prokaryotes) or the Kozak sequence (for eukaryotes), is necessary for the initiation of translation of mRNA. The element is typically located 3' to the promoter and 5' to the coding sequence of the polypeptide to be synthesized. 30   The Shine-Dalgarno sequence is varied but is typically a polypurine (i.e., having a high A-G content). Many Shine-Dalgarno sequences have been identified, each of which can be readily synthesized using methods set forth above and used in a prokaryotic vector. The 35   Kozak sequence typically includes sequences immediately before and after the initiating codon. A preferred

- 8 -

Kozak sequence is one that is associated with a high efficiency of initiation of translation at the AUG start codon.

5                   In those cases where it is desirable for NAT polypeptide to be secreted from the host cell, a signal sequence may be used to direct the polypeptide out of the host cell where it is synthesized. Typically, the signal sequence is positioned in the coding region of  
10 nucleic acid sequence, or directly at the 5' end of the coding region. Many signal sequences have been identified, and any of them that are functional in the selected host cell may be used here. Consequently, the signal sequence may be homologous or heterologous to  
15 the polypeptide. Additionally, the signal sequence may be chemically synthesized using methods referred to above.

                  After the vector has been constructed and a  
20 nucleic acid has been inserted into the proper site of the vector, the completed vector may be inserted into a suitable host cell for amplification and/or polypeptide expression.

25                   As mentioned, host cells may be prokaryotic (such as *E. coli*) or eukaryotic (such as a yeast cell, an insect cell, or a vertebrate cell). The host cell, whether it be yeast or some other host, when cultured under appropriate conditions can synthesize NAT, which  
30 can subsequently be collected from the culture medium (if the host cell secretes it into the medium) or directly from the host cell producing it (if it is not secreted). After collection, NAT polypeptide can be purified using methods such as molecular sieve  
35 chromatography, affinity chromatography, and the like.

- 9 -

Selection of the host cell will depend in large part on whether the manner in which the host cell is able to "fold" NAT into its native secondary and tertiary structure (e.g., proper orientation of disulfide bridges, etc.) such that biologically active material is prepared by the cell. However, even where the host cell does not synthesize biologically active material, it may be "folded" after synthesis using appropriate chemical conditions, such as ones that are known to those skilled in the art. In either case, proper folding can be inferred from the fact that biologically active material has been obtained.

Suitable host cells or cell lines may be mammalian cells, such as Chinese hamster ovary cells (CHO) or 3T3 cells. The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art. Other suitable mammalian cell lines are the monkey COS-1 and COS-7 cell lines, and the CV-1 cell line. Further exemplary mammalian host cells include primate cell lines and rodent cell lines, including transformed cell lines. Normal diploid cells, cell strains derived from *in vitro* culture of primary tissue, as well as primary explants, are also suitable. Candidate cells may be genotypically deficient in the selection gene, or may contain a dominantly acting selection gene. Still other suitable mammalian cell lines include but are not limited to, HeLa, mouse L-929 cells, 3T3 lines derived from Swiss, Balb-c or NIH mice, BHK or HaK hamster cell lines.

Also useful as host cells are bacterial cells. For example, the various strains of *E. coli* (e.g., HB101, DH5a, DH10, and MC1061) are well-known as

- 10 -

host cells in the field of biotechnology. Various strains of *B. subtilis*, *Pseudomonas* spp., other *Bacillus* spp., *Streptomyces* spp., and the like, may also be employed. Additionally, many strains of yeast  
5 cells known to those skilled in the art are also available as host cells for expression of the polypeptide of the present invention. Also, where desired, insect cells may be utilized as host cells. See, for example, Miller et al., *Genetic Engineering*,  
10 Volume 8, pages 277-298 (1986).

Insertion (also referred to as "transformation" or "transfection") of the vector into the selected host cell may be accomplished using such  
15 methods as calcium phosphate, electroporation, microinjection, lipofection or the DEAE-dextran method. The method selected will in part be a function of the type of host cell to be used. These methods and other suitable methods are well known to the skilled artisan,  
20 and are set forth, for example, in Sambrook et al., above.

The host cells containing the vector may be cultured using standard media well known to the skilled  
25 artisan. The media will usually contain all nutrients necessary for the growth and survival of the cells. Suitable media for culturing *E. coli* cells are, for example, Luria Broth (LB) and/or Terrific Broth (TB). Suitable media for culturing eukaryotic cells are RPMI  
30 1640, MEM, DMEM, all of which may be supplemented with serum and/or growth factors as required by the particular cell line being cultured. A suitable medium for insect cultures is Grace's medium supplemented with yeastolate, lactalbumin hydrolysate and/or fetal calf  
35 serum, as necessary.

- 11 -

Typically, an antibiotic or other compound useful for selective growth of the transformed cells only is added as a supplement to the media. The compound to be used will be dictated by the selectable  
5 marker element present on the plasmid with which the host cell was transformed. For example, where the selectable marker element is kanamycin resistance, the compound added to the culture medium will be kanamycin.

10 The amount of NAT produced in the host cell can be evaluated using standard methods known in the art. Such methods include, without limitation, Western blot analysis, SDS-polyacrylamide gel electrophoresis, non-denaturing gel electrophoresis, HPLC separation,  
15 immunoprecipitation, and/or activity assays such as DNA binding gel shift assays.

If NAT is secreted from the host cells other than gram-negative bacteria, the majority will likely  
20 be found in the cell culture medium. If NAT is secreted from gram-negative bacteria, it will to some degree be found in the periplasm. If NAT is not secreted, it will be present in the cytoplasm.

25 For intracellular NAT, the host cells are typically first disrupted mechanically. For NAT having a periplasmic location, either mechanical disruption or osmotic treatment can be used to release the periplasmic contents into a buffered solution. NAT  
30 polypeptide is then isolated from this solution. Purification from solution can thereafter be accomplished using a variety of techniques. If NAT has been synthesized so that it contains a tag such as hexahistidine or other small peptide at either its  
35 carboxyl or amino terminus, it may essentially be purified in a one-step process by passing the solution

- 12 -

through an affinity column where the column matrix has a high affinity for the tag or for the polypeptide directly (i.e., a monoclonal antibody). For example, polyhistidine binds with great affinity and specificity to nickel, thus an affinity column of nickel (such as the Qiagen nickel columns) can be used for purification. (See, for example, Ausubel et al., editors, Current Protocols in Molecular Biology, above).

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Where, on the other hand, the polypeptide has no tag and no antibodies are available, other well known procedures for purification can be used. Such procedures include, without limitation, ion exchange chromatography, molecular sieve chromatography, reversed phase chromatography, HPLC, native gel electrophoresis in combination with gel elution, and preparative isoelectric focusing ("Isoprime" machine/technique, Hoefer Scientific). In some cases, two or more of these techniques may be combined to achieve increased purity.

Especially preferred for use in the production of NAT are yeast cells, and most advantageously those of the yeast genus known as *Pichia* (e.g., *Pichia pastoris*), because of the greater efficiency of refolding compared to, for instance, bacterial cells such as *E. coli*. Suitable recombinant methods of expression for this yeast strain are described in United States Patent Nos. 4,855,231 (Stroman et al.), 4,812,405 (Lair et al.), 4,818,700 (Cregg et al.), 4,885,242 (Cregg) and 4,837,148 (Cregg), the disclosures of which are incorporated herein by reference.

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- 13 -

Notably, *Pichia* cells can also be used to express fibrolase with similar efficiency from DNA molecules encoding this metalloproteinase, and such a method constitutes an additional aspect of the present invention. Fibrolase is a known metalloproteinase which has been described in the scientific and patent literature; see Randolph et al., and European Patent Application No. 0 323 722, cited above. Typically, the fibrolase to be expressed will be of SEQ ID NO: 5, which is encoded by the cDNA molecule of SEQ ID NO: 6 (or variants thereof encoding the same amino acid sequence). The expression of fibrolase in such a system will typically involve a DNA molecule of SEQ ID NO: 7, which encodes "prepro" sequence (nucleotides 1-783) in addition to the "mature" polypeptide (nucleotides 784-1392).

Chemically modified versions of NAT in which the polypeptide is linked to a polymer or other molecule to form a derivative in order to modify properties are also included within the scope of the present invention. For human therapeutic purposes especially, it may be advantageous to derivatize NAT in such a manner by the attachment of one or more other chemical moieties to the polypeptide moiety. Such chemical moieties may be selected from among various water soluble polymers. The polymer should be water soluble so that the NAT polypeptide to which it is attached is miscible in an aqueous environment, such as a physiological environment. The water soluble polymer may be selected from the group consisting of, for example, polyethylene glycol, copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1,3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids

- 14 -

(either homopolymers or random or non-random copolymers (see further below regarding fusion molecules), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propylene glycol homopolymers, polypropylene  
5 oxide/ethylene oxide co-polymers, polyoxyethylated polyols, polystyrenemaleate and polyvinyl alcohol. Polyethylene glycol propionaldehyde may have advantages in manufacturing due to its stability in water.

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The polymer may be of any molecular weight, and may be branched or unbranched. For polyethylene glycol, the preferred molecular weight is between about 2 kilodaltons (kDa) and about 100 kDa (the term "about"  
15 indicating that in preparations of polyethylene glycol, some molecules will weigh more, some less, than the stated molecular weight) for ease in handling and manufacturing. Other sizes may be used, depending on the desired therapeutic profile (e.g., the duration of  
20 sustained release desired, the effects, if any on biological activity, the ease in handling, the degree or lack of antigenicity and other known effects of the polyethylene glycol on a therapeutic protein).

25

The number of polymer molecules so attached may vary, and one skilled in the art will be able to ascertain the effect on function. One may mono-derivatize, or may provide for a di-, tri-, tetra- or some combination of derivatization, with the same or  
30 different chemical moieties (e.g., polymers, such as different weights of polyethylene glycols). The proportion of polymer molecules to NAT polypeptide molecules will vary, as will their concentrations in the reaction mixture. In general, the optimum ratio  
35 (in terms of efficiency of reaction in that there is no excess unreacted polypeptide or polymer) will be

- 15 -

determined by factors such as the desired degree of derivatization (e.g., mono, di-, tri-, etc.), the molecular weight of the polymer selected, whether the polymer is branched or unbranched, and the reaction  
5 conditions.

The chemical moieties should be attached to NAT with consideration of effects on functional or antigenic domains of the polypeptide. There are a  
10 number of attachment methods available to those skilled in the art. See, for example, EP 0 401 384 (coupling PEG to G-CSF), and Malik et al., Experimental Hematology, Volume 20, pages 1028-1035 (1992) (reporting the pegylation of GM-CSF using tresyl  
15 chloride). By way of illustration, polyethylene glycol may be covalently bound through amino acid residues via a reactive group, such as, a free amino or carboxyl group. Reactive groups are those to which an activated polyethylene glycol molecule (or other chemical moiety)  
20 may be bound. The amino acid residues having a free amino group may include lysine residues and the N-terminal amino acid residue. Those having a free carboxyl group may include aspartic acid residues, glutamic acid residues, and the C-terminal amino acid  
25 residue. Sulfhydryl groups may also be used as a reactive group for attaching the polyethylene glycol molecule(s) (or other chemical moiety). Preferred for manufacturing purposes is attachment at an amino group, such as at the N-terminus or to a lysine group.  
30 Attachment at residues important for receptor binding should be avoided if receptor binding is desired.

One may specifically desire N-terminally chemically modified derivatives. Using polyethylene  
35 glycol as an illustration, one may select from a variety of polyethylene glycol molecules (by molecular

- 16 -

weight, branching, etc.), the proportion of polyethylene glycol molecules to polypeptide molecules in the reaction mixture, the type of pegylation reaction to be performed, and the method of obtaining the selected N-terminally pegylated NAT. The method of obtaining the N-terminally pegylated preparation (i.e., separating this moiety from other monopegylated moieties if necessary) may be by purification of the N-terminally pegylated material from a population of pegylated NAT molecules. Selective N-terminal chemical modification may be accomplished by reductive alkylation which exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization. See PCT application WO 96/11953, published April 25, 1996. Under the appropriate reaction conditions, substantially selective derivatization of NAT at the N-terminus with a carbonyl group containing polymer is achieved. For example, one may selectively N-terminally pegylate NAT by performing the reaction at a pH which allows one to take advantage of the  $pK_a$  differences between the  $\epsilon$ -amino group of the lysine residues and that of the  $\alpha$ -amino group of the N-terminal residue of the polypeptide. By such selective derivatization, attachment of a polymer to a polypeptide is controlled: the conjugation with the polymer takes place predominantly at the N-terminus of the polypeptide and no significant modification of other reactive groups, such as lysine side chain amino groups, occurs. Using reductive alkylation, the polymer may be of the type described above, and should have a single reactive aldehyde for coupling to the polypeptide. Polyethylene glycol propionaldehyde, containing a single reactive aldehyde, may be used.

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- 17 -

NAT or chemically modified derivatives in accordance with the invention may be formulated for *in vivo* administration, and most preferably via intra-thrombus (i.e., via localized delivery directly to the site of the clot in the blood vessel, e.g., as by catheter). Systemic delivery is normally not preferred due to the likelihood that innate  $\alpha_2$  macroglobulin in the general circulation may complex with NAT to prevent interaction with fibrin or fibrinogen, thus impairing clot lysis. However, there may be instances where larger amounts of NAT can be used which exceed the circulating levels of  $\alpha_2$  macroglobulin, thus enabling systemic administration and delivery. In general, encompassed within the invention are pharmaceutical compositions comprising effective amounts of NAT together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. By "effective amount" is meant amount sufficient to produce a measurable biological effect (i.e., a thrombolytically effective amount which effects lysis of the blood clot or clots being treated).

Typically, NAT will be in highly purified form, and any pharmaceutical composition being used as the delivery vehicle will normally be presterilized for use, such as by filtration through sterile filtration membranes.

One skilled in the art will be able to ascertain effective dosages by administration and observing the desired therapeutic effect. Particular effective doses within this range will depend on the particular disorder or condition being treated, as well as the age and general health of the recipient, and can be determined by standard clinical procedures. Where

- 18 -

possible, it will be desirable to determine the dose-response curve of the pharmaceutical composition first *in vitro*, as in bioassay systems, and then in useful animal model systems *in vivo* prior to testing in humans. The skilled practitioner, considering the therapeutic context, type of disorder under treatment, and other applicable factors, will be able to ascertain proper dosing without undue effort. Typically, a practitioner will administer the NAT composition until a dosage is reached that achieves the desired effect (i.e., lysis of the blood clot). The composition may be administered as a single dose, or as two or more doses (which may or may not contain the same amount of polypeptide) over time, or on a continuous basis.

NAT may also be used to generate antibodies in accordance with standard methods. The antibodies may be polyclonal, monoclonal, recombinant, chimeric, single-chain and/or bispecific, etc. To improve the likelihood of producing an immune response, the amino acid sequence of NAT can be analyzed to identify portions of the molecule that may be associated with increased immunogenicity. For example, the amino acid sequence may be subjected to computer analysis to identify surface epitopes, such as in accordance with the method of Hope and Woods, Proceedings of the National Academy of Science USA, Volume 78, pages 3824-3828 (1981).

Various procedures known in the art can be used for the production of polyclonal antibodies which recognize epitopes of NAT. For the production of antibody, various host animals can be immunized by injection with the polypeptide, including but not limited to rabbits, mice, rats, etc. Various adjuvants

- 19 -

may be used to increase the immunological response, depending on the host species, including but not limited to Freund's, mineral gels such as aluminum hydroxide (alum), surface active substances such as  
5 lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as Bacille Calmette-Guerin and *Corynebacterium parvum*.

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For the preparation of monoclonal antibodies directed toward NAT, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the  
15 hybridoma technique originally developed by Kohler and Milstein which is described in Nature, Volume 256, pages 495-497 (1975), as well as the trioma technique, the human B-cell hybridoma technique described by Kozbor et al. in Immunology Today, Volume 4, page 72  
20 (1983), and the EBV-hybridoma technique to produce monoclonal antibodies described by Cole et al. in "Monoclonal Antibodies and Cancer Therapy", Alan R. Liss, Inc., pages 77-96 (1985), are all useful for preparation of monoclonal antibodies in accordance with  
25 this invention.

The antibodies of this invention can be used therapeutically, such as to bind to and thereby neutralize or inhibit excess amounts of NAT *in vivo*  
30 after administration. The antibodies can further be used for diagnostic purposes, such as in labeled form to detect the presence of NAT in a body fluid, tissue sample or other extract, in accordance with known  
35 diagnostic methods.

- 20 -

Description of Specific Embodiments

The invention is further illustrated in the following examples.

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Example 1Derivation of NAT Sequence

- 10           An effective way to produce fibrolase is to express it initially as preprofibrolase in which cleavage by the protease kex-2 occurs at the junction of the "prepro" and "mature" regions to yield biologically active material ("mature" fibrolase).
- 15   From this design, the synthesis and processing of the preprofibrolase leads to secretion of mature fibrolase into the culture medium. The actual sequence at the cleaved junction is (...TKR↓QORF...).
- 20           Kex-2 is an endoprotease that cleaves after two adjacent basic amino acids, in this case lysine(K)-arginine(R). Mature fibrolase expressed from DNA having the above mentioned sequence revealed that the expected N-terminal glutamine (Q) residue had in fact
- 25   undergone deamidation and cyclization to generate pyroglutamic acid (E). This chemical modification was deemed undesirable, since peptides with an N-terminal cyclized glutamine (pyroglutamic acid) residue fail to react in the Edman degradation procedure for amino acid
- 30   sequencing. Accordingly, both of the glutamine (Q) residues at the N-terminus in the sequence for mature fibrolase were deleted, resulting in an N-terminal arginine (R) residue. Since kex-2 cleaves after two adjacent basic amino acids as mentioned, it was
- 35   anticipated that the sequence (...KRRF...) would

- 21 -

present an ambiguous site for kex-2 cleavage. Accordingly, the N-terminal arginine (R) residue (shown underlined above) was replaced with a serine (S) residue to result in the sequence (...KRSF...). The choice of serine was based on the need to introduce an amino acid which facilitates kex-2 cleavage when it occurs on the C-terminal side of the hydrolysis site. Rholam et al., European Journal of Biochemistry, Volume 227, pages 707-714 (1995).

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As a result, the DNA sequence for preprofibrolase was modified by site-directed mutagenesis at the N-terminal coding region for mature fibrolase to substitute the codons for "QQR" with a codon for "S", using a standard PCR protocol, thus resulting in preproNAT having the amino acid sequence of SEQ ID NO: 3. The oligonucleotides used to prime the PCR reactions are listed below, and their homology with the target sequence is also shown. Initially, two PCR reactions were carried out using oligos 1 and 4 as one primer pair and oligos 2 and 3 as another primer pair, both with DNA of the parent gene as the template. The DNA products of these two reactions (601 and 815 nucleotides in length) were purified by agarose gel electrophoresis and combined to serve as a template in a second round of PCR using oligos 1 and 2 as the primer pair. This final PCR product (1372 nucleotides in length) was cleaved with restriction endonucleases *XhoI* and *NotI*. The digest was deproteinized with phenol/chloroform and DNA precipitated. A portion of the recovered DNA was ligated into the plasmid pPICZ $\alpha$  (Invitrogen, Carlsbad, CA, Catalog No. VI95-20), which had been similarly cleaved with restriction endonucleases *XhoI* and *NotI*, enzymatically dephosphorylated, and deproteinized with

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- 22 -

phenol/chloroform. All subsequent steps were carried out according to the Invitrogen Pichia Expression Kit manual (Invitrogen Corp., Catalog No. K1710-01). The ligation reaction products were transformed into *E. coli* by electroporation and selected for survival on zeocin-containing solid media. The plasmid was isolated and the profibrolase region was confirmed by DNA sequencing. The plasmid was linearized by cleaving with restriction endonuclease *PmeI* and then transformed into *Pichia pastoris* GS115<sup>his<sup>+</sup></sup>. The GS115 strain is normally *his<sup>-</sup>*, so the *his<sup>+</sup>* genotype was restored by transformation with a DNA source carrying the wild type version of the *his4* gene. Alternatively, a *his<sup>+</sup>* strain can be obtained commercially from Invitrogen Corp. (X-33 cell line, Catalog No. C180-00). Integrants were selected as zeocin-resistant colonies. Candidate clones were induced in methanol-containing media, and the broth was assayed for NAT production on 4-20% PAGE, using Coomassie staining.

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The oligonucleotides used for site-directed PCR mutagenesis were as follows:

25 Oligo 1 5'-TACTATTGCCAGCATTGCTGC-3' (SEQ ID NO: 8)

Oligo 2 5'-GCAAATGGCATTCTGACATCC-3' (SEQ ID NO: 9)

Oligo 3 (SEQ ID NO: 10)

5'-TCCAATTAACTTGACTAAGAGATCTTTCCCACAAAGATACGTAC-3'

30

Oligo 4 (SEQ ID NO: 11)

5'-GTACGTATCTTTGTGGGAAAGATCTCTTAGTCAAGTTTAATTGG-3'

The location of these oligonucleotides is shown below in relation to the double-stranded DNA sequence (SEQ ID NO: 12 coding or sense strand, SEQ ID

35

- 23 -

NO: 13 complementary or antisense strand) and  
corresponding amino acid sequence (SEQ ID NO: 14) of  
fibrolase (including the prepro region) being modified  
to create NAT. The N-terminal and C-terminal regions  
5 of mature fibrolase are indicated by underlining of  
terminal amino acid sequences (QQR $\overline{F}$  and LNKP). The  
N-terminal region (QQR $\overline{F}$ ) is the one being modified (to  
substitute S for QQR). For oligos 3 and 4, below,  
dashed lines are inserted to denote the location of the  
10 omitted codons encoding for residues QQ in the  
N-terminal region of fibrolase.

ATGAGATTTCC $\overline{TTCAATTTT}$ ACTGCTGTTTATTTCGCAGCATCCTCCGCATTAGCTGCT  
-----+-----+-----+-----+-----+-----+  
15 TACTCTAAAGGAAGTTAAAAATGACGACAAAATAAGCGTCGTAGGAGGCGTAATCGACGA

M R F P S I F T A V L F A A S S A L A A

CCAGTCAACACTACAACAGAAGATGAAACGGCACAAATTCGGGCTGAAGCTGTTCATCGGT  
20 -----+-----+-----+-----+-----+-----+  
GGTCAGTTGTGATGTTGTCTTCTACTTTGCCGTGTTAAGGCCGACTTCGACAGTAGCCA

P V N T T T E D E T A Q I P A E A V I G

TACTCAGATTTAGAAGGGGATTTTCGATGTTGCTGTTTGGCCATTTTCCAACAGCACAAAT  
25 -----+-----+-----+-----+-----+-----+  
ATGAGTCTAAATCTTCCCTAAAGCTACAACGACAAAACGGTAAAAGGTTGTCGTGTTTA

Y S D L E G D F D V A V L P F S N S T N  
30

Oligo 1 5'-TACTATTGCCAGCATTGCTGC-3'

AACGGGTTATTGTTTATAAATACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTA  
-----+-----+-----+-----+-----+-----+  
TTGCCCAATAACAAATATTTATGATGATAACGGTCGTAACGACGATTTCTTCTCCCAT  
35

N G L L F I N T T I A S I A A K E E G V

- 24 -

XhoI

|

TCTCTCGAGAAAAGAGAGGCTGAAGCTTCTTCTATTATCTTGGAACTCTGGTAACGTTAAC  
-----+-----+-----+-----+-----+-----+  
5 AGAGAGCTCTTTTCTCTCCGACTTCGAAGAAGATAATAGAACCCTTAGACCATTGCAATTG  
  
S L E K R E A E A S S I I L E S G N V N  
  
GATTACGAAGTTGTTTATCCAAGAAAGGTCCTCCAGTTCCTAGGGGTGCTGTTCAACCA  
10 -----+-----+-----+-----+-----+-----+  
CTAATGCTTCAACAAATAGGTTCTTCCAGTGAGGTCAAGGATCCCCACGACAAGTTGGT  
  
D Y E V V Y P R K V T P V P R G A V Q P  
  
15 AAGTACGAAGATGCCATGCAATACGAATCAAGGTTAACAGTGAACCAGTTGTCTTGCAC  
-----+-----+-----+-----+-----+-----+  
TTCATGCTTCTACGGTACGTTATGCTTAAGTTCCAATTGTCACTTGGTCAACAGAACGTG  
  
K Y E D A M Q Y E F K V N S E P V V L H  
20 -----+-----+-----+-----+-----+-----+  
TTGGAAAAAACAAGGTTTGTCTCTGAAGATTACTCTGAAACTCATTACTCCCCAGAT  
-----+-----+-----+-----+-----+-----+  
AACCTTTTTTTGTTTCCAAACAAGAGACTTCTAATGAGACTTTGAGTAATGAGGGGTCTA  
  
25 L E K N K G L F S E D Y S E T H Y S P D  
  
GGTAGAGAAATTACTACTTACCCATTGGGTGAAGATCACTGTTACTACCATGGTAGAATC  
-----+-----+-----+-----+-----+-----+  
CCATCTCTTTAATGATGAATGGGTAACCCACTTCTAGTGACAATGATGGTACCATCTTAG  
30 -----+-----+-----+-----+-----+-----+  
G R E I T T Y P L G E D H C Y Y H G R I  
  
GAAAACGATGCTGACTCCACTGCTTCTATCTCTGCTTGTAACGGTTTGAAGGGTCATTTT  
-----+-----+-----+-----+-----+-----+  
35 CTTTTGCTACGACTGAGGTGACGAAGATAGAGACGAACATTGCCAAACTTCCCAGTAAAG  
  
E N D A D S T A S I S A C N G L K G H F  
  
AAGTTGCAAGGTGAAATGTACTTGATTGAACCATTGGAATTGTCCGACTCTGAAGCCCAT  
40 -----+-----+-----+-----+-----+-----+  
TTCAACGTTCCACTTTACATGAACTAAGTTGGTAACCTTAACAGGCTGAGACTTCGGGTA  
  
K L Q G E M Y L I E P L E L S D S E A H

- 25 -

GCTGTCTACAAGTACGAAAACGTCGAAAAGGAAGATGAAGCCCCAAAGATGTGTGGTGT  
-----+-----+-----+-----+-----+-----+-----+  
CGACAGATGTTTCATGCTTTTGCAGCTTTTCCTTCTACTTCGGGGTTTCTACACACCACAA  
5 A V Y K Y E N V E K E D E A P K M C G V

Oligo 3 5'-TCCAATTAACTTGACTAAG  
ACCCAAAACCTGGGAATCATATGAACCAATCAAGAAGGCCCTTCCAATTAACTTGACTAAG  
10 -----+-----+-----+-----+-----+-----+-----+  
TGGGTTTTGACCCTTAGTATACTTGGTTAGTTCTTCCGGAAGGTTAATTTGAACTGATTC  
Oligo 4 3'-GGTTAATTTGAACTGATTC

T Q N W E S Y E P I K K A F Q L N L T K  
15 AGA-----TCTTTCCCAAAAGATACGTAC-3'  
AGACAACAAAGATTCCCACAAAGATACGTACAGCTGGTTATCGTTGCTGACCACCGTATG  
-----+-----+-----+-----+-----+-----+-----+  
TCTGTGTTTCTAAGGGTGTTCCTATGCATGTCGACCAATAGCAACGACTGGTGGCATA  
20 TCT-----AGAAAGGGTGTTCCTATGCATG-5'

R Q Q R F P Q R Y V Q L V I V A D H R M

AACACTAAATACAACGGTGACTCTGACAAAATCCGTCAATGGGTGCACCAAATCGTCAAC  
25 -----+-----+-----+-----+-----+-----+-----+  
TTGTGATTTATGTTGCCACTGAGACTGTTTTAGGCAGTTACCCACGTGGTTTAGCAGTTG

N T K Y N G D S D K I R Q W V H Q I V N

ACCATTAACGAAATCTACAGACCACTGAACATCCAATTCACCTTTGGTTGGTTTGGAAATC  
30 -----+-----+-----+-----+-----+-----+-----+  
TGGTAATTGCTTTAGATGTCTGGTGACTTGTAGGTTAAGTGAAACCAACCAACCTTTAG

T I N E I Y R P L N I Q F T L V G L E I  
35 TGGTCCAACCAAGATTTGATCACCGTTACTTCTGTATCCCACGACACTCTGGCATCCTTC  
-----+-----+-----+-----+-----+-----+-----+  
ACCAGGTTGGTTCTAACTAGTGGCAATGAAGACATAGGGTGCTGTGAGACCGTAGGAAG

40 W S N Q D L I T V T S V S H D T L A S F

- 26 -

GGTAACTGGCGTGAAACCGACCTGCTGCGTCGCCAACGTCATGATAACGCTCAACTGCTG  
-----+-----+-----+-----+-----+-----+  
CCATTGACCGCACTTTGGCTGGACGACGACGCGGTTGCAGTACTATTGCGAGTTGACGAC

5 G N W R E T D L L R R Q R H D N A Q L L

ACCGCTATCGACTTCGACGGTGATACTGTTGGTCTGGCTTACGTTGGTGGCATGTGTCAA  
-----+-----+-----+-----+-----+-----+  
TGGCGATAGCTGAAGCTGCCACTATGACAACCAGACCGAATGCAACCACCGTACACAGTT

10 T A I D F D G D T V G L A Y V G G M C Q

CTGAAACATTCTACTGGTGTTATCCAGGACCACTCCGCTATTAACCTGCTGGTTGCTCTG  
-----+-----+-----+-----+-----+-----+  
GACTTTGTAAGATGACCACAATAGGTCCTGGTGAGGCGATAATTGGACGACCAACGAGAC

15 L K H S T G V I Q D H S A I N L L V A L

ACCATGGCACACGAACTGGGTCATAACCTGGGTATGAACCACGATGGCAACCAAGTGTAC  
-----+-----+-----+-----+-----+-----+  
TGGTACCGTGTGCTTGACCCAGTATTGGACCCATACTTGGTGCTACCGTTGGTCACAGTG

20 T M A H E L G H N L G M N H D G N Q C H

TGCGGTGCAAACCTCCTGTGTTATGGCTGCTATGCTGTCCGATCAACCATCCAAACTGTTT  
-----+-----+-----+-----+-----+-----+  
ACGCCACGTTTGAGGACACAATACCGACGATACGACAGGCTAGTTGGTAGGTTTGACAAG

25 C G A N S C V M A A M L S D Q P S K L F

TCCGACTGCTCTAAGAAAGACTACCAGACCTTCCTGACCGTTAACAACCCGAGTGTATC  
-----+-----+-----+-----+-----+-----+  
AGGCTGACGAGATTCTTCTGATGGTCTGGAAGGACTGGCAATTGTTGGGCGTCACATAG

30 S D C S K K D Y Q T F L T V N N P Q C I

35

NotI

CTGAACAAACCGTAAGCGGCCCGCCAGCTTTCTAGAACAAAACTCATCTCAGAAGAGGAT  
-----+-----+-----+-----+-----+-----+  
GACTTGTGTTGGCATTGCGCCGGCGGTCGAAAGATCTTGTTTTGAGTAGAGTCTTCTCCTA

40

L N K P \* A A A S F L E Q K L I S E E D

- 27 -

CTGAATAGCGCCGTCGACCATCATCATCATCATCATGAGTTTGTAGCCTTAGACATGAC  
-----+-----+-----+-----+-----+-----+  
GACTTATCGCGGCAGCTGGTAGTAGTAGTAGTAGTAACCTCAAACATCGGAATCTGTACTG  
5 L N S A V D H H H H H H \* V C S L R H D  
  
TGTTCTCAGTTCAAGTTGGGCACTTACGAGAAGACCGGTCTTGCTAGATTCTAATCAAG  
-----+-----+-----+-----+-----+-----+  
10 ACAAGGAGTCAAGTTCAACCCGTGAATGCTCTTCTGGCCAGAACGATCTAAGATTAGTTC  
  
C S S V Q V G H L R E D R S C \* I L I K  
  
AGGATGTCAGAATGCCATTTGCCTGAGAGATGCAGGCTTCATTTTTGATACTTTTTTATT  
15 -----+-----+-----+-----+-----+-----+  
TCCTACAGTCTTACGGTAAACGGACTCTCTACGTCCGAAGTAAAAACTATGAAAAAATAA  
CCTACAGTCTTACGGTAAACG-5' Oligo 2  
  
R M S E C H L P E R C R L H F \* Y F F I -  
20

## Example 2

### Expression of NAT in *Pichia pastoris*

25 When attempts were made to express the DNA  
for NAT in *E. coli*, very poor refolding and a  
requirement for dilute conditions reduced the  
purification efficiency. These and other  
30 considerations led to the usage of *Pichia pastoris*, a  
yeast species, as the host cell. A culture of selected  
clones of *Pichia pastoris* which had been transfected  
with prepro NAT cDNA (SEQ ID NO: 4) was inoculated into  
500 ml of the following inoculation growth medium:  
35

- 28 -

Per liter of batch medium

|   |                             |                      |
|---|-----------------------------|----------------------|
|   | Yeast extract               | 30.0 g               |
|   | Potassium phosphate dibasic | 17.2 g               |
| 5 | Glucose                     | 20.0 g               |
|   | Biotin                      | 0.004 g              |
|   | Water                       | to 1 liter           |
|   | Phosphoric acid, 85%        | to adjust pH to 6.00 |

- 10                   The transfected *P. pastoris* cells were incubated at 30°C in a shaker for about 30 to 32 hours. About 1% (w/v) of the resulting culture was used to inoculate a 10-liter fermentor. The fermentor contained sterilized basal salts and glucose (below).
- 15   Twelve milliliters per liter of PTM4 salts (PTM4 is a trace metals solution containing cupric sulfate pentahydrate, sodium iodide, manganese sulfate monohydrate, sodium molybdate dihydrate, boric acid, cobaltous chloride hexahydrate, zinc chloride, ferrous
- 20   sulfate heptahydrate, d-biotin, sulfuric acid and purified water) were added per liter of batch medium after fermentor sterilization. The fermentation growth temperature was 30°C. The fermentor pH was controlled with ammonium hydroxide and phosphoric acid at pH 6.00.
- 25   Zinc from the zinc salts added to the medium becomes incorporated into NAT as part of the metalloproteinase structure.

Basal salts per liter of batch medium

|    |                                     |         |
|----|-------------------------------------|---------|
| 30 | Phosphoric acid, 85%                | 26.7 ml |
|    | Calcium sulfate                     | 0.93 g  |
|    | Potassium sulfate                   | 18.2 g  |
|    | Magnesium sulfate-7H <sub>2</sub> O | 14.9 g  |
| 35 | Potassium hydroxide                 | 4.13 g  |

- 29 -

|         |            |
|---------|------------|
| Glucose | 30.0 g     |
| Water   | to 1 liter |

The batch culture was grown until the glucose  
5 was completely consumed (17 to 20 hours). Then a fed-  
batch phase was initiated. The fed-batch phase media  
consisted of glucose and 12 ml of PTM4 salts per liter.  
The induction feed consisted of glucose, 25% methanol,  
and 12 ml of PTM4 salts per liter. At induction, the  
10 temperature of the reactor was shifted to 20°C. The  
induction phase lasted 60 to 75 hours. The conditioned  
media were harvested and the cellular debris was  
discarded.

15

Example 3Purification of NAT from *Pichia pastoris*

The yeast broth (conditioned media less  
20 cellular debris) from Example 2 was clarified and the  
pH and conductivity were adjusted to 6.5 and 10-20  
mS/cm, respectively. The broth was loaded onto an  
immobilized metal affinity resin that had been charged  
with copper (Cu) and equilibrated with phosphate  
25 buffered saline (PBS). The resin was washed with PBS  
and eluted with an imidazole gradient (0-100 mM) in  
PBS. Fractions containing "mature" NAT (SEQ ID NO: 1)  
were pooled and diluted until the conductivity was less  
than 1.5 mS/cm, pH 6.4. The diluted pool was loaded  
30 onto an SP Sepharose resin (Amersham Pharmacia Biotech,  
Inc., Piscataway, NJ) that had been equilibrated with  
10 mM 2-(N-morpholino)ethanesulfonic acid (MES). The  
column was washed with MES and eluted with a NaCl  
gradient (0-500mM) in MES. Fractions containing NAT  
35 were pooled and stored.

- 30 -

Example 4Thrombolysis in Acute Thrombosis of Rat  
5      Carotid Artery; Comparison of NAT with Urokinase

To demonstrate that "mature" NAT (SEQ ID NO:  
1) is biologically active and functionally unique,  
acute pharmacology studies were conducted in rats where  
10 focal injury to one of the carotid arteries was created  
by applying anodal current. This injury produces an  
occlusive thrombus which generally forms within fifteen  
minutes. Once the thrombus was formed, the artery was  
observed for a period of thirty minutes to assure that  
15 the carotid occlusion was stable. Then heparin and  
aspirin were administered intravenously to prevent  
further propagation of the thrombus. The animals were  
then treated with an intraarterial infusion of test  
material. Blood flow through the carotid artery was  
20 monitored during the delivery of test material so that  
successful clot lysis could be detected and the time at  
which clot lysis occurred could be noted. The  
percentage of experiments where clot lysis occurred was  
noted and group means were calculated for only those  
25 experiments where clot lysis was successful. As a  
measure of the hemorrhagic potential of the test  
material, any blood that was shed from the surgical  
site was collected with gauze swabs. The swabs were  
placed in a detergent solution to solubilize red blood  
30 cells and release hemoglobin, which was then quantified  
spectrophotometrically. Shed hemoglobin was used to  
calculate a volume of blood loss. Test data are  
reported in the Table below.

- 31 -

Table 1

Incidence of Clot Lysis, Time to Clot  
Lysis and Surgical Blood Loss (Mean  $\pm$  std. dev.)

5

|                                 | INCIDENCE<br>OF LYSIS<br>(%) | TIME TO<br>LYSIS<br>(min) | BLOOD LOSS<br>(ml) |
|---------------------------------|------------------------------|---------------------------|--------------------|
| SALINE<br>(n=6)                 | 0%<br>(0 of 6)               | N/A                       | 0.10<br>$\pm$ 0.23 |
| Urokinase<br>25U/min<br>(n=15)  | 33%<br>(5 of 15)             | 55.3<br>$\pm$ 15.9        | 1.06<br>$\pm$ 1.59 |
| Urokinase<br>250U/min<br>(n=15) | 86%<br>(13 of 15)            | 33.5<br>$\pm$ 15.3        | 1.43<br>$\pm$ 1.45 |
| NAT 2mg<br>(n=14)               | 78%<br>(11 of 14)            | 6.3<br>$\pm$ 5.8          | 0.96<br>$\pm$ 0.77 |

These studies establish that NAT is  
biologically active in an animal model of *in vivo* clot  
10 lysis. Further, clot lysis was achieved in a markedly  
reduced amount of time and with less blood loss from  
the surgical site, in comparison with urokinase. Thus,  
the activity profile of NAT can be distinguished from  
the plasminogen activator class of thrombolytic agents  
15 (represented by urokinase) in that clot lysis with NAT  
occurs more rapidly and with reduced hemorrhagic  
complications.

The fibrinolytic activity of NAT is comparable to  
20 that of fibrolase. In addition, as mentioned above the  
stability of the N-terminus of NAT results in a more

- 32 -

homogeneous end product upon recombinant expression,  
which is a distinct advantage (i.e., the N-terminus  
will not change over time resulting in a mixture of  
different forms, thus making the polypeptide more  
5 stable).

- 33 -

CLAIMS

1. A fibrinolytically active polypeptide  
5 having the amino acid sequence of SEQ ID NO: 1.
2. The polypeptide of claim 1 which has  
been made by recombinant expression in yeast.
- 10 3. A nucleic acid molecule encoding the  
polypeptide of SEQ ID NO: 1.
4. The nucleic acid molecule of claim 3  
which has the sequence of SEQ ID NO: 4.
- 15 5. An expression vector comprising  
expression regulatory elements operatively linked to a  
nucleic acid molecule according to claim 3.
- 20 6. An expression vector according to claim  
5 in which the nucleic acid molecule has the nucleotide  
sequence of SEQ ID NO: 4.
7. A host cell transformed or transfected  
25 with the nucleic acid molecule of claim 3.
8. A transformed or transfected host cell  
according to claim 7 which is a prokaryotic or  
eukaryotic cell.
- 30 9. A transformed or transfected prokaryotic  
cell according to claim 8 which is a yeast cell.
10. A transformed or transfected yeast cell  
35 according to claim 9 which is *Pichia pastoris*.



- 34 -

11. A method for the production of NAT, comprising culturing host cells containing a DNA molecule encoding said metalloproteinase operatively linked to regulatory elements for stimulating the expression thereof under conditions such that the DNA molecule is expressed, and isolating the expressed metalloproteinase from the host cell culture.
12. The method of claim 11 in which the DNA molecule is of SEQ ID NO: 4.
13. The method of claim 11 in which the host cells are yeast cells.
14. The method of claim 13 in which the yeast cells are of *Pichia pastoris*.
15. A method for the production of a metalloproteinase fibrinolytic agent selected from the group consisting of fibrolase and NAT, comprising culturing *Pichia* host cells containing a DNA molecule encoding said metalloproteinase operatively linked to regulatory elements for stimulating the expression thereof under conditions such that the DNA molecule is expressed, and isolating the expressed metalloproteinase from the host cell culture.
16. The method of claim 15 in which the *Pichia* host cell is *Pichia pastoris*.
17. A method for the treatment of thrombosis in a mammal comprising administering to the mammal a thrombolytically effective amount of NAT.
18. An antibody against the polypeptide of claim 1.

- 35 -



19. The antibody of claim 17 which is a  
monoclonal antibody.