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(54) Title: HYALURONIDASE FROM THE *HIRUDINARIA MANILLENSIS*, ISOLATION, PURIFICATION AND RECOMBINANT METHOD OF PRODUCTION

(57) Abstract: The present invention relates to the isolation, purification and characterization of a hyaluronidase which derives from the tropical leech *Hirudinaria manillensis*. Therefore, according to this invention, the enzyme was called "manillase". The invention is furthermore concerned with the recombinant method of production of manillase which includes the disclosure of DNA and amino acid sequences as well as of expression vectors and host systems. Finally, the invention relates to the use of manillase for therapeutic purposes, for example, for the treatment of myocardial diseases, thrombotic events and tumors.

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## Hyaluronidase from the *Hirudinaria manillensis*, isolation, purification and recombinant method of production

The present invention relates to the isolation, purification and characterization of  
5 a novel hyaluronidase which derives from the tropical leech *Hirudinaria manillensis*. Therefore, according to this invention the new enzyme is called "manillase". The invention is furthermore concerned with the recombinant method of production of manillase which includes the disclosure of DNA and amino acid sequences as well as of expression vectors and host systems. Finally,  
10 the invention relates to the use of manillase for therapeutic purposes, for example, for the treatment of myocardial diseases, thrombotic events and tumors.

Hyaluronic acid or hyaluronan (HA) is a linear unbranched high molecular-weight ( $2-6 \times 10^6$ ) glycosaminoglycan, composed of a repeating disaccharide structure  
15 GlcNAc( $\beta$ 1-4)GlcUA. Its carboxyl groups are fully ionized in the prevailing pH of extracellular fluids, whether normal or pathological. HA belongs together with the chondroitin sulphates, keratan sulfates and heparins to the group of glycosaminoglycans (Jeanloz R. W., *Arthr Rheum.*, 1960, 3, 233-237). In contrast with other unmodified glycosaminoglycans (GAG), it has no sulfate substitution or  
20 covalently linked peptide, and its chain length and molecular weight are usually very much greater. HA is ubiquitously distributed in connective tissues and has been found in virtually all parts of the body after introduction of improved fixation method (Hellström S. et al., 1990, *Histochem. J.*, 22, 677-682) and the specific histochemical method with the use of hyaluronan-binding peptides (HABP). It is  
25 present during development and maturity in tissues of neuroectodermal origin as well.

The term hyaluronidase refers generally and according to this invention to an enzyme, which acts on hyaluronic acid, irrespective of activity towards other  
30 substrates.

Hyaluronidase was first isolated from microorganisms and later from mammalian testis which is now its main source (Meyer K. in *The Enzyme*, 1971, 307).

According to the reaction mechanism, hyaluronidases were divided into three main groups.

In the first group microbial enzymes are combined that act on their substrates by  $\beta$ -elimination producing  $\Delta$ -4,5-unsaturated disaccharides. The enzyme must  
5 therefore be named hyaluronate lyases, EC 4.2.99.1.

The second group, hyaluronoglucosaminidase or testicular-type hyaluronidase (EC 3.2.1.35) acts as an endo-N-acetyl- $\beta$ -D-hexosaminidase degrading HA to smaller fragments, in the first place tetrasaccharide with the hexosamine moiety  
10 at the free reducing end. Enzymes with similar properties to the testis hyaluronidase have been obtained from tadpoles, snake venom, bee venom, numerous animal tissues, human serum and other sources. It is well known that hyaluronidase from testis has also transglycosylase activity (Weissman B. et al., *J. Biol. Chem.*, 1954, 208, 417-429). The enzymes belonging to this group of  
15 hyaluronidases exhibit enzymatic activity not only towards hyaluronate but also towards chondroitin-4-sulfate, chondroitin-6-sulfate, chondroitin and dermatan sulfate.

The third group consists of hyaluronoglucuronidase (EC 3.2.1.36), which acts as  
20 an endo- $\beta$ -glucuronidase. This enzyme was isolated from the *Hirudo medicinalis* leeches (Yuki H. & Fishman W.H.; *J. Biol. Chem.* 1963, 238, 1877-79) and is absolutely specific for HA. Chondroitin sulfate, dermatan and heparin are not substrates for this hyaluronidase. It degrades only hyaluronic acid to tetrasaccharide with the glucuronic acid at the free reducing end (Linker A. et al.,  
25 *J. Biol. Chem.*, 1960, 235, 924-27). Opposite to mammalian endo- $\beta$ -glucosaminidases, heparin has no influence on the activity of this leech hyaluronidase. Therefore, it can be coadministered to a patient together with a heparin and its derivatives extensively used as anticoagulants. A hyaluronic acid specific endo-beta-glucuronidase (called "Orgelase") from species (*Poecilobdella*  
30 *granulosa*) of the sub-family *Hirudinariinae* (including the genera *Hirudinaria*, *Illebdella*, *Poecilobdella*, *Sanguisoga*) of buffalo leeches was disclosed in EP 0193 330 having a molecular weight of about 28,5.

Hyaluronidases have many practical in vivo and in vitro applications. Intravenous administration of hyaluronidase has been proposed for treatment of myocardial infarction (Kloner R.A et al., *Circulation*, 1978, 58, 220-226; Wolf R.A. et al., *Am. J. Cardiol.*, 1984, 53, 941-944; Taira A. et al., *Angiology*, 1990, 41, 1029-1036).

5 Myocardial infarction represents a common form of non-mechanical injury; namely severe cell damage and death, caused in this instance by sudden cellular hypoxia. In an experimental myocardial infarction induced in rats (Waldenström A. et al., 1991, *J. Clin. Invest.*, 88, 1622-1628), HA content of the injured (infarcted area) heart muscle increased within 24 h to reach nearly three times  
10 normal after 3 days, and was accompanied by interstitial oedema. The relative water content of infarcted areas also increased progressively reaching a maximum value by day 3 and was strongly correlated with the HA accumulation. The same association of increased HA content with oedema has been observed in experimental heart and renal transplant rejection (Hällgren R. et al., *J. Clin.*  
15 *Invest.*, 1990, 85, 668-673; Hällgren R. et al., *J. Exp. Med.*, 1990, 171, 2063-2076) in rejection of human renal transplants (Wells A. et al. *Transplantation*, 1990, 50, 240-243), lung diseases (Bjermer A. et al., *Brit. Med. J.*, 1987, 295, 801-806) and in idiopathic interstitial fibrosis (Bjermer A. et al., *Thorax*, 1989, 44, 126-131). All these studies provide not only evidence of increased HA in acute  
20 inflammation, but demonstrate its part in the local retention of fluid mainly responsible for the tissue swelling and influencing both the mechanical and electrophysiological functions of heart.

These results can explain the mechanism of the action of hyaluronidases used in  
25 clinical trials. It was reported that hyaluronidase treatment limited cellular damage during myocardial ischemia in rats, dogs and man (Maclean D. et al. *Science*, 1976, 194, 199). The degradation of the HA can be followed by the reduction of tissue water accumulation, reduction of the tissue pressure and finally better perfusion.

30

It has been shown that hyaluronidases as well as hyaluronidase containing extracts from leeches can be used for other therapeutic purposes. Thus, hyase therapy, alone or combined with cyclosporine, resulted in prolonged graft survival (Johnsson C. et al. *Transplant Inter.* in press). Hyases ("spreading factor") in the

broadest sense are used to increase the permeability of tissues for enhancing the diffusion of other pharmacological agents (e.g. in combination with cytostatics in the treatment of cancer tumors). Furthermore, it could be demonstrated that hyaluronidases are useful in tumor therapy acting as angiogenesis inhibitor and as an aid to local drug delivery in the treatment of tumors, for the treatment of glaucoma and other eye disorders and as adjunct to other therapeutic agents such as local anaesthetics and antibiotics. A general overview of the therapeutic use and relevance is given in the review article of Farr et al. (1997, Wiener Medizinische Wochenschrift, 15, p. 347) and literature cited therein.

Therefore, there is a need for an active compound such as hyaluronidase. However, the known and available hyaluronidases are either not stable (hyaluronidase from *Hirudo medicinalis*, Linker et. al., 1960, J. Biol. Chem. 235, p. 924; Yuki and Fishman, 1963, J. Biol. Chem. 238, p. 1877) or they show a rather low specific activity (EP 0193 330, Budds et al., 1987, Comp. Biochem. Physiol., 87B, 3, p. 497). Moreover, none of the known hyaluronidases are available in recombinant form which is an essential prerequisite for intensive commercial use.

This invention discloses now for the first time a new hyaluronidase which was isolated and purified from *Hirudinaria manillensis* as well as a recombinant version of said enzyme obtained by bioengineering techniques.

Thus, it is an object of this invention to provide a purified protein isolated from the leech species *Hirudinaria manillensis* having the biological activity of a hyaluronidase which is not influenced in its activity by heparin and characterized in that it has a molecular weight of 53 – 60 kD dependent on glycosylation. The new protein, which is called "manillase", is glycosylated in its native form having a molecular weight of ca. 58 kD ( $\pm 2$  kD) and four glycoforms. However, the non-glycosylated protein is object of the invention as well, obtainable by enzymatic or chemical cleavage of the sugar residues according to standard techniques. The non-glycosylated enzyme of the invention has a molecular weight of about 54 ( $\pm 2$ ) as measured by SDS-PAGE.

Direct comparison shows that the hyaluronidase disclosed in EP 0193 330 ("orgelase") has under the same conditions a molecular weight of about 28 and contains a lot of impurities such as hemoglobin.

Native manillase according to this invention has a pH optimum of 6.0 – 7.0, an  
5 isoelectric point of 7.2 – 8.0 and has the amino acid sequence depicted in Fig. 7.

Surprisingly manillase obtained by a preparative purification procedure (see below) has an extremely high specific activity of 100 – 150, preferably of 110 – 140 (WHO) kU/mg protein whereas the specific activity of orgelase is about 1,2  
10 kU/ mg only. Moreover, orgelase has a lower pH optimum (5.2 - 6.0) as compared with manillase. Manillase is not influenced, like orgelase, by heparin.

Furthermore it is an object of the invention to provide a process for isolating and purifying manillase comprising the following steps

- 15 (i) homogenization of heads of leeches of the species *Hirudinaria manillensis* with an acid buffer and centrifugation,
- (ii) ammonium sulfate precipitation of the supernatant of step (i),
- (iii) cation exchange chromatography,
- (iv) concanavalin A affinity chromatography
- 20 (v) hydrophobic interaction chromatography
- (vi) affinity chromatography on matrices coated with hyaluronic acid fragments
- (vii) gel permeation chromatography, and optionally
- (viii) enzymatic or chemical deglycosylation of the purified protein.

25 The process steps disclosed above guarantee that the protein according to the invention can be obtained with such a high biological enzyme activity. Therefore, it is a further object of this invention to provide a protein having the biological activity of a hyaluronidase which is not influenced in its activity by heparin and having a molecular weight of 53 – 60 dependent on glycosylation which is  
30 obtainable by the process steps indicated above and in the claims and which has preferably a specific enzyme activity of > 100 kU/ mg protein. The term "unit" relates below and above to "international units" (IU).

The invention discloses a process of making recombinant manillase which includes respective DNA molecules, vectors and transformed host cells.

Therefore, it is an object of this invention to provide a DNA sequence coding for a protein having the properties of native manillase.

- 5 It could be also shown, that at least three further clones with slightly different DNA sequences could be selected which are coding for proteins with manillase (hyaluronidase) properties having slightly different amino acid sequences.

- The specified clones have the DNA sequences depicted in Fig. 8, 9 and 10  
10 (upper sequence) which are an object of this invention too as well as expression vectors containing said sequences and host cells which were transformed with said vectors.

- In addition, it is object of this invention to provide a recombinant protein with the  
15 biological activity of a hyaluronidase and a molecular weight of 55 – 59 kD dependent on glycosylation having any amino acid sequence depicted in Fig. 8, 9 and 10 (lower sequence) or a sequence which has a homology to said sequences of at least 80%. The term "manillase" includes all these proteins having the above-specified properties.

20

- The native as well as the recombinant protein(s) may be used as a medicament which can be applied to patients directly or within pharmaceutical compositions. Thus, it is a further aspect of this invention to provide a recombinant or native protein as defined above and below applicable as a medicament and a respective  
25 pharmaceutical composition comprising said protein and a pharmaceutically acceptable diluent, carrier or excipient therefor.

- The pharmaceutical compositions of the invention may contain additionally further active pharmaceutical compounds of a high diversity. Preferred agents are  
30 anticoagulants which do not inhibit or influence the biological and pharmacological activity of the protein according to the invention. Such anticoagulants can be, for example, heparin, hirudin or dicoumarin, preferably, heparin. Thus, it is an object of the present invention to provide a pharmaceutical

composition comprising additionally a pharmacologically active compound, preferably heparin.

In connection with use in human or veterinary therapy the protein according to this invention acts preferably as dispersal agent ("spreading" factor) or supports penetration through tissue and skin. Thus, manillase can be used as an adjunct of other substances (such as an local anaesthetic) e.g. in the field of chemotherapy of tumors, for treatment of disorders and diseases with respect to acute myocardial ischemia or infarction, for treatment of glaucoma and other eye disorders, e.g. to improve the circulation of physiological fluids in the eye, for treatment of skin and tissue grafts to remove congestion and improve circulation, as drug delivery system through the skin, membranes, other tissue, as an agent to remove the hyaluronic acid capsule surrounding certain pathogenic microorganisms or certain tumors and cancerous tissues, and as an inhibitor of angiogenesis which can be used as anti-thrombotic and anti-tumor agent.

Therefore, the use of manillase as defined above and below in the manufacture of a medicament for treating especially myocardial, cardiovascular and thrombotic disorders and tumors is an object of this invention.

20

As used herein, the term "pharmaceutically acceptable carrier" means an inert, non toxic solid or liquid filler, diluent or encapsulating material, not reacting adversely with the active compound or with the patient. Suitable, preferably liquid carriers are well known in the art such as sterile water, saline, aqueous dextrose, sugar solutions, ethanol, glycols and oils, including those of petroleum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil and mineral oil.

25

The formulations according to the invention may be administered as unit doses containing conventional non-toxic pharmaceutically acceptable carriers, diluents, adjuvants and vehicles which are typical for parenteral administration.

30

The term "parenteral" includes herein subcutaneous, intravenous, intra-articular and intratracheal injection and infusion techniques. Also other administrations such as oral administration and topical application are suitable. Parenteral



compositions and combinations are most preferably administered intravenously either in a bolus form or as a constant fusion according to known procedures. Tablets and capsules for oral administration contain conventional excipients such as binding agents, fillers, diluents, tableting agents, lubricants, disintegrants, and wetting agents. The tablets may be coated according to methods well known in the art.

Oral liquid preparations may be in the form of aqueous or oily suspensions, solutions, emulsions, syrups or elixirs, or may be presented as a dry product for reconstitution with water or another suitable vehicle before use. Such liquid preparations may contain conventional additives like suspending agents, emulsifying agents, non-aqueous vehicles and preservatives. Topical applications may be in the form of aqueous or oily suspensions, solutions, emulsions, jellies or preferably emulsion ointments.

Unit doses according to the invention may contain daily required amounts of the protein according to the invention, or sub-multiples thereof to make up the desired dose. The optimum therapeutically acceptable dosage and dose rate for a given patient (mammals, including humans) depends on a variety of factors, such as the activity of the specific active material employed, the age, body weight, general health, sex, diet, time and route of administration, rate of clearance, enzyme activity (units/mg protein), the object of the treatment, i. e., therapy or prophylaxis and the nature of the disease to be treated.

Therefore, in compositions and combinations such as with anticoagulants like heparin in a treated patient (in vivo) a pharmaceutical effective daily dose of the protein of this invention (manillase) is between about 0.01 and 100 mg/kg body weight (based on a specific activity of 100 kU/mg), preferably between 0.1 and 10 mg/kg body weight. According to the application form one single dose may contain between 0.5 and 10 mg of manillase.

The concentration of e.g. heparin when administered together with manillase is typically 500 – 4000 U (IU) over one day, however, may be increased or diminished if necessary.

The purification of manillase of the invention was achieved as described in detail in the examples. Table 1 depicts a preparative purification scheme of manillase. Table 2 shows the process of enrichment of the protein according to the invention  
5 and Table 3 indicates the comparison of manillase with known leech hyaluronidases.

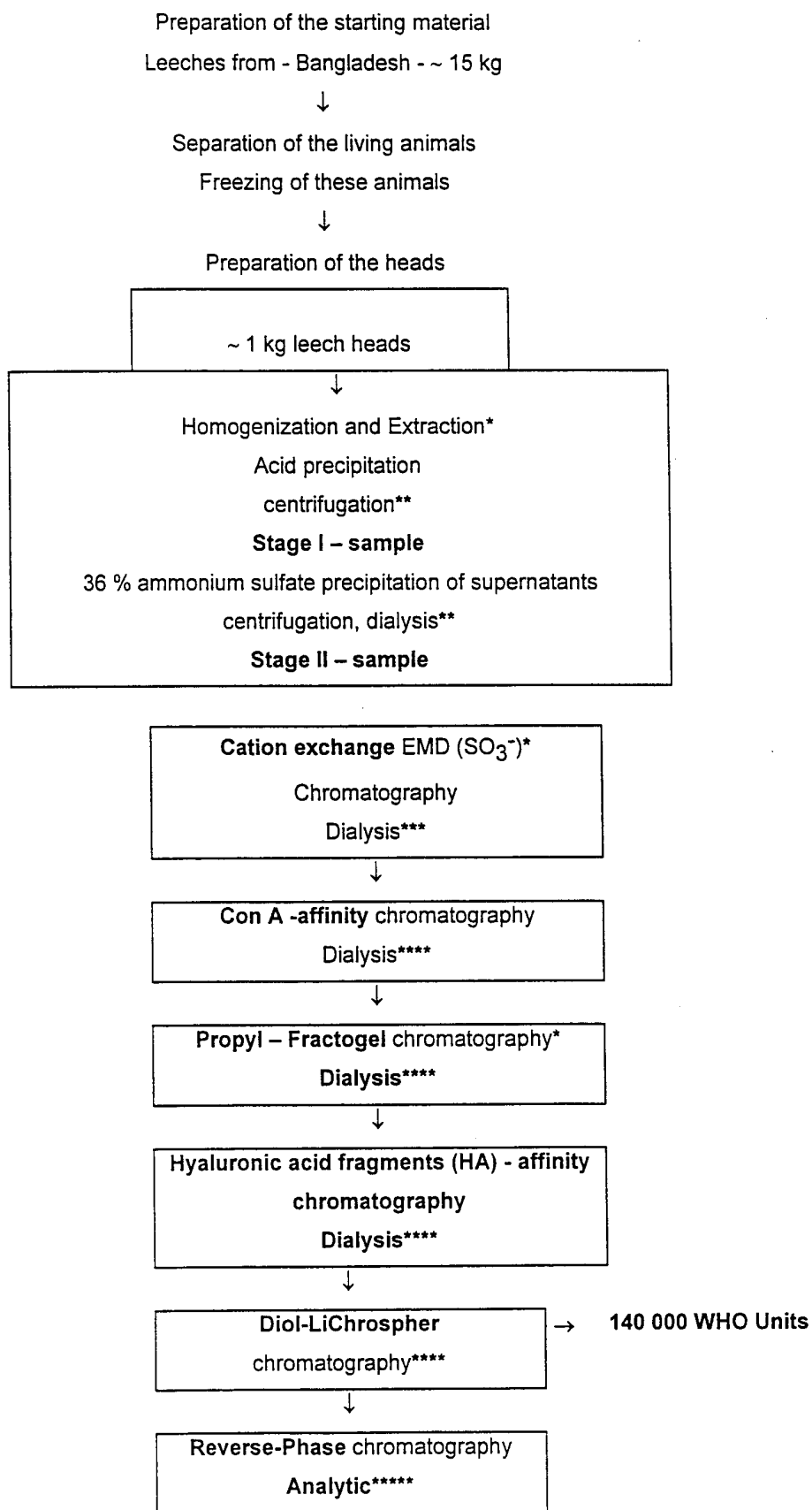
An enzyme, named manillase, cleaving hayaluronic acid has been isolated from the heads of *Hirudinaria manillensis* leeches and purified to homogeneity. This  
10 hyaluronidase was purified using acid-extraction, ammoniumsulfate precipitation, followed by successive chromatography on cation exchanger, Concanavalin A-Sepharose, Propyl-Fractogel, Hyaluronan fragments-Sepharose and Diol-LiChrospher columns. The hyaluronan fragments were prepared by the cleavage of the native hyaluronan with the aid of bovine testes hyaluronidase. After  
15 purification and characterization of the fragments, the affinity matrices were prepared as indicated below. Such affinity matrices were applied for the first time for purification of the hyaluronidase. This high-performance chromatography is a technique for fast and efficient purification of hyaluronan binding proteins. The recovery of enzyme activity after each step of purification was reasonably high.  
20 The results of the three independent preparative purifications were comparable. They resulted in highly active samples possessing between 20 to 160 kU/mg dependent on the degree of purification. In comparison experiments known hyaluronidases were isolated as indicated in the prior art and their properties were compared with the protein according to this invention (Tab. 3).

25

The hyaluronidase purified according to the scheme of Tab. 1 differs from other leech hyaluronidases described by other authors. A similar molecular weight was obtained under non-dissociating conditions (any  $\beta$  mercaptoethanol), indicating that manillase is a single subunit enzyme in common with a wide range of  
30 hyaluronidase preparations from mammalian sources. This final preparation is a single subunit enzyme (Fig. 1) of apparent molecular weight  $58 \pm 2$  determined with the aid of MALDI, with isoelectric point of 7,2 to 8,0.

**Tab. 1:** *Preparative purification of manillase*

5



10

**Tab.2:** Purification of manillase (enrichement) from 1 kg of leech heads

Step of purification	Total protein Mg	Total activity kU	% recovery	Specific activity U/mg	Purification (fold)
<b>Stage I</b> supernatant after extraction and acid precipitation	31 700	633.3	100	20	1
<b>Stage II</b> supernatant after 36% ammonium sulfate precipitation	9 530	443.3	70	45	2.25
<b>Cation exchange</b> chromatography	426.7	332.5	52.5	770	38.5
<b>Con A affinity -</b> chromatography	41.0	166.2	26.2	4.000	200
<b>Propyl-Fractogel</b> chromatography	11.9	133.0	21.0	11 000	550
<b>Hyaluronic acid</b> fragments-Sepharose affinity chromatography	1.9	66.4	10.5	35 000	1 750
<b>Diol-LiChrospher</b>	0.307	33.2	5.2	108 000	5 400

**Tab.3:** Comparison of manillase with known leech hyaluronidases

	<b>"Manillase"</b> Hirudinaria manillens. Invention	<b>Hyaluronidase</b> H. medicinalis comparison experiment	<b>Hyaluronidase</b> H. medicinalis Linker et al.; (J.Biol.Chem, 1960)	<b>"Orgelase"</b> P. granulosa EP 0 193 330 Budds et al.
specific activity WHO (IU) units/mg	140 000	~20 000 semipurified	≤100	≤100
homogeneity SDS-PAGE MALDI	1 protein homogenous 4 glycoforms	Mixture of proteins	no results available	mixture of many proteins main impurity: hemoglobin
molecular weight	58,3 kD ± 2 kD	n. d.	not reported	28,5 ± 3 kD
amino acid sequence	determined	n. d.	not reported	not determined
pH optimum	6.0 - 7.0	6.0 - 7.0	not reported	5,2 - 6.0
pI	7.5 - 8,0	n. d.	n. d.	n. d.
hydrophobicity	binding to Propyl- HIC at 2 M ammonium sulfate	no binding to Propyl-HIC at 2 M ammonium sulfate		
activity reduction by heparin	no influence	not determined	no influence	no influence
<b>Stability</b>				
at +4°C	stable after 7 days ~ 75% activity retained	Unstable 100% loss of activity after 7 days incubation		
at +37°C	stable after 7 days ~ 60% activity retained	Unstable 100% loss of activity after 7 days incubation		relatively stable
stability at +37°C in the presence of the dog's serum	stable after 7 days ~100% activity retained	Unstable 100% loss of activity after 1 day incubation	not reported	not tested

The asterisks in the tables mean information on activity determination and biochemical characterization (\* - \*\*\*\*\*).

The methods of activity determination and biochemical characterization used depend of the concentration of manillase in the analyzed samples. Therefore,  
5 they were successively extended by the appropriate techniques in the successive steps of purification.

- \* - Activity determination - turbidity reduction test
- \*\* - Activity determination -turbidity reduction test
- Protein content determination (E<sub>280</sub>, Pierce BCA method)
- 10 - SDS - PAGE (SDS - Polyacrylamide Gel Electrophoresis)
- Hemoglobin determination
- \*\*\* - Activity determination -turbidity reduction test
- Protein content determination (E<sub>280</sub>, Pierce BCA method)
- SDS - PAGE - Western Blot (anti human hemoglobin antibody)
- 15 \*\*\*\* - Activity determination -turbidity reduction test
- Protein content determination (E<sub>280</sub>, Pierce BCA method)
- SDS - PAGE - Western Blot anti human hemoglobin antibody,
- SDS - PAGE - Western Blot anti Con A antibody
- SDS - PAGE - Western Blot - anti peptide antibodies
- 20 \*\*\*\*\* - MALDI
- Protein content determination (Pierce BCA method)
- SDS - PAGE - Western Blot - anti peptide antibodies

25 Binding of manillase to Concanavalin A shows that this hyaluronidase is a glycoprotein, whose sugar components are terminated with  $\alpha$ -D-mannopyranosyl or  $\alpha$ -D-glucopyranosyl and sterically related residues. Manillase-active samples showed two bands with almost identical RF values in SDS-PAGE. Longer SDS-PAGE and different running conditions were used for better separation of the  
30 bands. In these experiments two additional, weaker bands could be detected (Fig. 2). The N-terminal part all of them (30 amino acids) was individually sequenced and showed again no difference in the N-terminus. Following deglycosylation with the endo-F-glycosidase (PNGase) it was observed that all four bands resulted in a single band, with a reduction in MW of about 3.

Therefore, it is quite likely that the observed differences in electrophoretic mobility are due to differences in the glycosylation pattern of manillase molecules. The neuraminidase, O-endo-glycosidase and neuraminidase plus O-glycosidase treatments have no influence on the molecular weight of the purified enzyme (Fig. 3). These results have shown that manillase contains at least one N-linked oligosaccharide chain. The O-linked carbohydrate chains could not be detected with the method used.

As the concluding purification step, the RP-chromatography was carried out. Although the enzymatic activity could not be detected any more, the salts and peptide protease inhibitors could be removed (Fig. 4). The fractions containing protein were characterized further with the help of MALDI. The molecular weight of manillase determined with the aid of MALDI was 58,3.

Heparin has no influence on the activity of this hyaluronidase (Fig. 5). Manillase is many fold more stabile than *Hirudo medicinalis* hyaluronidase (Fig. 6). Moreover, the samples of partly purified manillase showed very high stability in the dogs and rats plasma within the -20 to + 37 range.

The preparation of HA-affinity matrices has been described in the literature (Tengblad A., *Biochim. Biophys. Acta*, 1979, **578**, 281-289). This HA-matrix was used for the purification of the cartilage hyaluronate binding proteins or proteoglycan protein-keratan sulfate core (Christner J. E., *Anal. Biochem.*, 1978, **90**, 22-32) from the same source. The HA-binding protein (HABP), purified with the aid of this affinity matrix, was used further in histochemical studies concerning the distribution of the hyaluronate receptors (Green S.J. et al., *J. Cell Science*, 1988, **89**, 145-156; Chan F. L. et al., *J. Cell. Biol.*, 1997, **107**, 289-301) or hyaluronan (Waldenström A. et al., 1991, *J. Clin. Invest.*, **88**, 1622-1628; Waldenström A. et al., *Eur. J. Clin. Invest.*, 1993, **23**, 277-282) in the tissues.

30

However, the method of the preparation of this gel developed in our laboratory enables one to produce gels of exactly defined concentration of HA-fragments (1 to 15 mg/ml). This, in turn, enables one to use such gels not only for purification of hyaluronan-binding proteins but also for their separation, by taking advantage

of their different affinity to hyaluronan. This selective separation can be controlled by using of HA-fragments of different length. Such separation will enable one to better characterization many receptors of biological relevance (e. g. in oncology)

- 5 HA-matrices prepared according to the method described can be applied for the:
- 1) purification of known HA-binding proteins
  - 2) purification of unknown HA-binding proteins
  - 3) identification of the new HA-binding proteins
  - 4) purification of hyaluronidases

10

HA-fragments obtained by the method described in the present invention can be characterized with the use of modern analytical methods (NMR, MALDI-MS) and applied in the research on protein-protein interactions. Furthermore, these fragments can be used in the research concerning angiogenesis and

15 neovascularization processes

Short description of the figures:

**Fig. 1:** SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE – CBB staining) of the protein standard, manillase sample (after Diol-LiChrospher chromatography).

20

1 – wide range protein standard

2 - Manillase, 4 $\mu$ g

3 - Orgelase, 6  $\mu$ g

4 – Hemoglobin, 40  $\mu$ g

25

**Fig. 2:** a) SDS-PAGE (CBB staining) and  
b) SDS-PAGE – Western blot of four manillase-active samples (lines 3-6) after HA - affinity chromatography. Rabbit P3-2A polyclonal anti-peptide antibody was used in this experiment.

**Fig. 3:** SDS-PAGE (CBB) of the following samples:

30

1- LW-MM – low weight molecular marker (BioRad)

2- Manillase

3- N-Glycosidase F (PNGase F)

4- Manillase after treatment with PNGase F

5- Manillase after treatment with O-glycosidase



6- Manillase after treatment with O-glycosidase and neuraminidase

7- O-glycosidase and neuraminidase

8- molecular weight marker (MWM-prestained BioRad)

**Fig. 4:** Reverse-Phase-Chromatography of

- 5 a) Ribonuclease standard  
b) manillase sample (specific activity 140 kU/mg)

**Fig. 5:** Influence of heparin on hyaluronidase activity of manillase ( - ○ - ) and bovine testes hyaluronidase ( - ● - )

X-axis: IU heparin; Y-axis: % activity left

10 **Fig. 6:** Stability measurement of hyaluronidases in buffer and plasma:

(a) manillase (4°C), (b) manillase (-20°C)

(c) manillase (37°C),

(d) bovine testes hyaluronidase (Y) and *Hirudo medicinalis* hyaluronidase (A)

15 X-axis: days of incubation; Y-axis: WHO (IU) units

**Fig. 7:** Amino acid sequence of native manillase obtained by sequencing of the isolated and purified protein from *Hirudinaria manillensis* according to the invention (corresponds to SEQ ID No. 1)

**Fig. 8:** Nucleotide (upper lines) and amino acid sequence of a recombinant manillase clone (clone 21); (corresponds to SEQ ID. Nos. 2, 3)

20 **Fig. 9:** Nucleotide (upper lines) and amino acid sequence of a recombinant manillase clone (clone 31); (corresponds to SEQ ID. Nos. 4, 5)

**Fig. 10:** Nucleotide (upper lines) and amino acid sequence of a recombinant manillase clone (clone 31); (corresponds to SEQ ID. Nos. 6, 7)

25 **Fig. 11:** *E. coli* expression vector for manillase

**Fig. 12:** Baculo donor plasmid for manillase

**Fig. 13:** Yeast expression vector for manillase

30 The invention is described in detail by the following examples. However, these examples do not limit the invention to the general materials, methods, physical parameters, compounds, biological materials, expression vectors and hosts etc. used in the experiments and indicated in the examples. If not otherwise mentioned standard techniques well known in the prior art and generally available material were used.

Example 1 (General Remarks):

A number of preliminary experiments were carried out using crude extracts of *Hirudinaria manillensis* in order to establish the purification procedure.

The following methods were chosen and verified: ammonium sulfate precipitation  
 5 procedure, cation and anion exchange chromatography, affinity chromatography with the aid of Heparin-Fractogel, Con A-Sepharose, Hydrophobic Interaction Chromatography (HIC) on Octyl-Sepharose, Propyl- Phenyl-, Butyl-Fractogel, preparative isoelectric focusing and preparative electrophoresis.

The results show that acid and ammonium precipitation, cation exchange, Con A-  
 10 Sepharose, Propyl-Fractogel HIC and Diol-LiChrospher and Hyaluronic acid fragments-Sepharose (HA-Sepharose) chromatography are suitable for the purification of the manillase. The HA-Sepharose matrix prepared in our laboratory was successfully used for the purification of this glycosidase.

All preparations were carried out in the cold unless otherwise mentioned.

15 The purification was done according to the scheme shown above (Tab. 1).

Example 2: - Preparation of the Starting Material for the Purification; Preparation of Leech Heads.

*Hirudinaria manillensis* leeches collected in Bangladesh were immediately shock-  
 20 frozen and then stored at -40° to -80°. They were decapitated in frozen state, the weight of the heads amounting to ca. 5% of the body.

Example 3: - Extraction Procedure of Manillase from Leech Heads

In a representative purification, 1 kg of frozen leech heads were homogenized in  
 25 a Waring Blender with 2500 ml of cold 0.1 M acetic acid buffer pH 4.0 containing 0,025% thimerosal and 17 mg/ml of trehalose (Merck KGaA, Art. No. 1.08216).

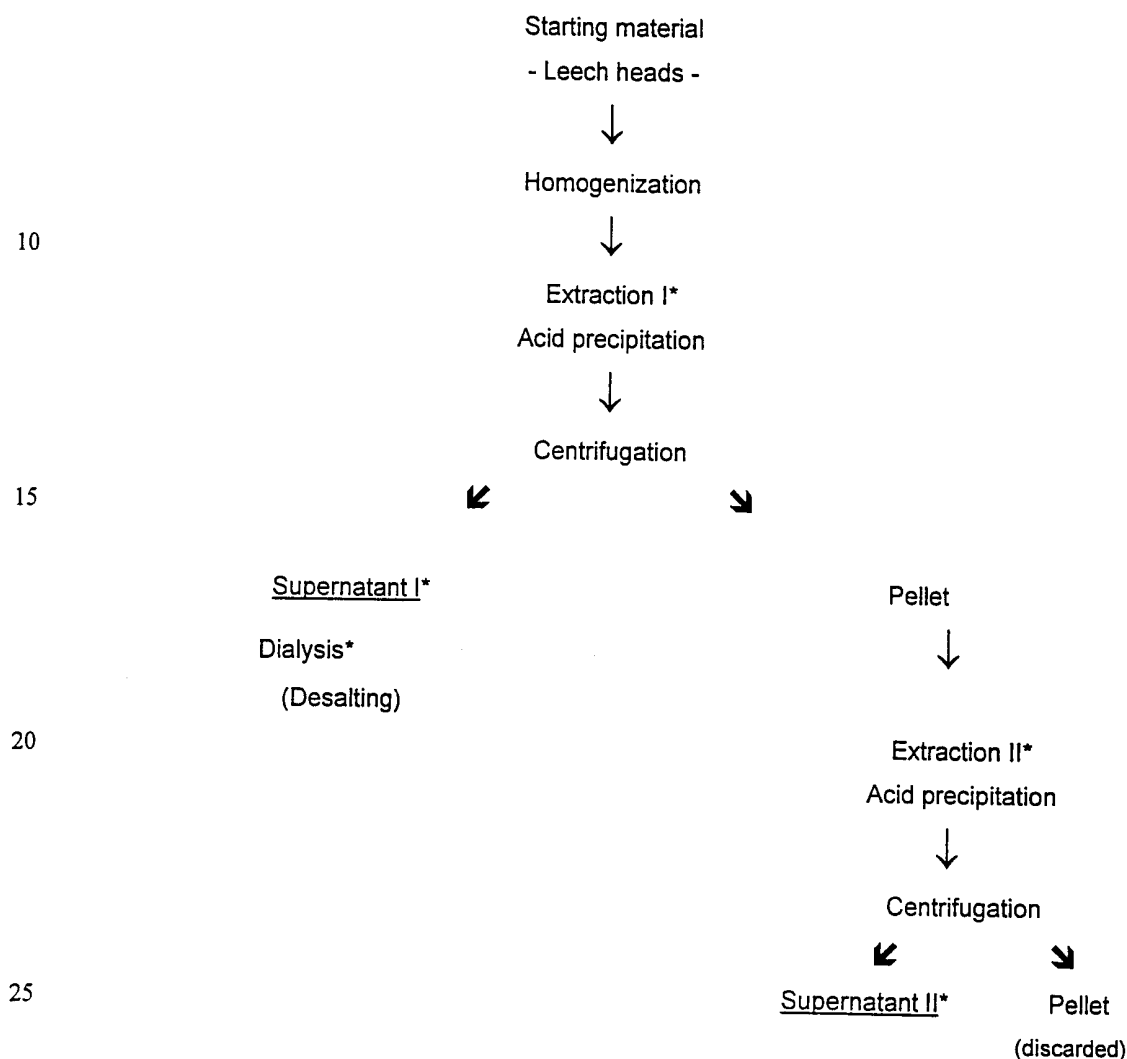
The homogenate was stirred gently and the following protease inhibitors were added immediately:

	1. PMSF	1.7 mg/ml	10.0 mM
30	2. Leupeptin	10.0 µg/ml	20.0 µM
	3. Pepstatin A	0.7 µg/ml	1 µM
	4. EGTA	380.35 µg/ml	1.0 mM
	5. p-APMSF	40.0 µg/ml	20.0 µM

Stirring was continued for 4 hour in the cold and centrifuged at 4900 rpm for 20 minutes. The supernatant solution (supernatant I) was collected and pooled with supernatant II subsequently obtained by extracting the tissues pellet.

The pooled supernatants represent Stage I material.

- 5 The procedure is summarized in the following scheme:



- \*Activity determination and biochemical characterization of the samples was performed with the aid of activity determination -turbidity reduction test and protein content determination (E<sub>280</sub>, Pierce BCA method, SDS - PAGE).
- It was impossible to measure the enzyme activity in the leech homogenate, because of the very high content of hemoglobins (measured with the hemoglobin determination kit, Merck KGaA, 13851) and other proteins. Moreover, the hyaluronidase activity could not be measured in the stage prior to the acid precipitation. The final specific activities (activity per mg of protein) of these

extracts were about 10-30 WHO Units. According to SDS-PAGE, the crude extracts contained large amounts of different proteins, the major ones having a molecular weight of ~120, 55 -60, 45, 31, 28, 22, 15 and 14-10.

5 Example 4: - Ammonium Sulfate Precipitation Procedure of the Stage I Material

Next, the ammonium sulfate precipitation procedure was chosen as the first step of the purification of manillase and resulted in a ~5-fold of enrichment of this enzyme.

Enzymatically inert material was precipitated from Stage I crude extract by adding  
10 slowly solid ammonium sulfate (Merck KGaA) to 36% w/v at +4°C. This mixture was stirred for 1 hour and centrifuged. The precipitate was discarded. The supernatant was dialyzed against running de-ionized water overnight, and 24 hours against 20 mM phosphate buffer pH 6.0. The final specific activities of these extracts were about 40 - 150 WHO Units. According to SDS-PAGE, the  
15 stage II extracts contain large amounts of different proteins.

Example 5: - Cation Exchange Chromatography

The cation exchanger was used in a batch adsorption mode. An enzyme-rich dialyzed sample (stage II) was incubated overnight with 1 l Fractogel EMD SO<sub>3</sub><sup>-</sup>  
20 650 (S) cation exchanger, Merck KGaA, Art. No. 16882. After the incubation was finished by centrifugation, the cation exchanger was washed with the buffer, centrifugated again and HPLC-Superformance column was filled with the gel. After washing the column with 20 mM phosphate buffer pH 4.9 the bound proteins were eluted from the column with the same sodium phosphate buffer pH 6.0  
25 containing a linear 0 to 1 M gradient of NaCl. Fractions were collected every 3 min (9 ml) and the absorbance at 280 nm was monitored. Manillase was eluted at 0,15 to 0,18 M NaCl concentrations. The activities and protein contents of all fractions were measured and the fractions were pooled and dialyzed overnight against 20 mM phosphate buffer pH 6,0 containing sodium azide and 17 mg/ml  
30 trehalose.

Determination of the concentration of proteins, specific activities of the "pools", and SDS-PAGE analysis were carried out. In spite of very good yields (activity) and high specific activity (WHO activity units per mg of protein, corresponds to IU), a mixture of many proteins was still shown by the results of SDS-PAGE

analysis of the samples. The cation exchange chromatography with the aid of Fractogel EMD SO<sub>3</sub><sup>-</sup>650 (S) ® (Merck KGaA, Germany) resulted in a very high purification factor of ~ 10 to 50. This step is very effective in reducing hemoglobin impurities. Moreover, we have found that the batch procedure was a very useful  
5 initial step for handling large volumes of stage II supernatant (5 - 16 l).

Example 6: - Concanavalin A -Sepharose Affinity Chromatography

The further purification of the enzyme-rich pools after cation exchanger was done with the aid of Con A lectin affinity chromatography. Commercially available Con  
10 A-Sepharose® from Pharmacia Biotech, Art. 17-0440-01, was washed with an acetic buffer 0.1 M + 0.5 M NaCl pH 8.0; 0.1 M boric acid + 0.1 % Triton X 100 pH 6.0 and finally with 0.1 M acetic buffer + 0.5 M NaCl pH 6.0. The sample was dialyzed overnight against 20 mM acetic buffer + 0.5 mM NaCl + 1 mM CaCl<sub>2</sub> + 1 mM MgCl<sub>2</sub> pH 6.0 + 1 mM MnCl<sub>2</sub>, applied at room temperature to a 1000 ml Con  
15 A column and eluted 2 h with the 510 ml of 100 mM acetic acid buffer + 0.5 M NaCl + 1 mM CaCl<sub>2</sub> + 1 mM MgCl<sub>2</sub> pH 6.0 + 1 mM MnCl<sub>2</sub>.

This was followed by desorption with the aid of the same buffer containing 0.5 M methyl- $\alpha$ -D-mannopyranoside. The elution was continuously monitored at 280 nm. The 3 ml fractions that had been collected were assayed for hyaluronidase  
20 activity. The active fractions were pooled and dialyzed overnight against 20 mM phosphate buffer pH 6.0 containing sodium azide and 17 mg/ml trehalose. Determination of the concentration of proteins, specific activities of the "pools", and SDS-PAGE analysis was carried out. This step was very effective in removing the rest of hemoglobin. The Con A chromatography resulted in a 4-10  
25 purification factor. This factor differed, depending on the quality of the starting material.

Example 7: - Propyl Fractogel Hydrophobic Interaction Chromatography

To hyaluronidase active Con A-pools ammonium sulfate were added to a final  
30 concentration of 2 M. The samples were then incubated 1 h at room temperature with 150 ml Propyl-Fractogel EMD Propyl 650 (S) ®, Merck KgaA, Germany, Art. No. 1.10085, equilibrated with 0.1 M phosphate buffer pH 7.0, containing 2 M ammonium sulfate. After the incubation was finished the gel was washed twice with the same buffer, and the HPLC-Superformance (2.6 cm x 60 cm) column

was prepared. The bound proteins were eluted with 0.1 M phosphate buffer pH 7.0. The 6 ml fractions were collected every 3 min, directly dialyzed against de-ionized water (2 - 3 h) and, then against 20 mM phosphate buffer pH 6.0. The fractions were assayed for hyaluronidase activity. The active fractions were  
5 pooled and dialyzed overnight against 20 mM phosphate buffer pH 6.0 containing sodium azide and 17 mg/ml trehalose. The protein and activity determination of the pools was carried out.

The purification factor at this chromatography step was about 3 to 5. A small amount of Con A released from the carrier gel in the previous step was removed  
10 together with other protein impurities.

Example 8: - Preparation of hyaluronic acid oligosaccharide affinity column

*(a) Hydrolysis of hyaluronan (HA) with bovine testes hyaluronidase*

Hyaluronic acid, 7 g was dissolved in 1,25 l of 0.1 M sodium acetate buffer  
15 containing 0.15 M NaCl and 0.5 mM EDTA, pH 5.2 by mixing overnight at 4°C in the presence of toluene. Thereafter pH of HA containing solution was adjusted to 5.2 and after warming up to 37°C, bovine testes hyaluronidase (Merck KGaA; 700 WHO units/mg) was added. For 7 g of HA, 210 mg of enzyme dissolved immediately before use in 50 ml of the above buffer were used. Hydrolysis was  
20 allowed to proceed for 30 min at 37°C with constant stirring, and terminated by heating for 5 min at 100°C in a boiling water bath. The reaction mixture was clarified through centrifugation for 30 min at 10 000 g, denatured protein containing sediment was discarded and supernatant filtered through 0.2 µm filter, on which a glass fiber prefilter was placed. Clarified solution containing HA  
25 oligosaccharides (HAOS) was fractionated by filtration through tree Diaflo ultrafiltration membrane (Amicon) with different molecular cut off values as follows.

*(b) Fractionation of HAOS by ultrafiltration*

HAOS-containing solution from the previous step was filtered through 30 YM  
30 Diaflo ultrafiltration membrane. Retentate was saved for other studies while filtrate was subjected to the second ultrafiltration through 10 YM Diaflo ultrafiltration membrane. Again, retentate was saved for other studies while the solution passing through 10 YM was subjected to the last ultrafiltration through 3 YM Diaflo membrane. Thereafter, retentate containing HA-OS, about 10 ml of the

solution, was used for further purification. This fraction: HAOS 3-10 was purified as follows and further used for coupling to Sepharose.

*(c) Purification of HAOS 3-10*

HA-OS 3-10 were purified (desalted) on Biogel P2 ® column. This column (4 cm  
5 x 100 cm) was packed with Biogel 2 medium ®, 200 – 400 mesh (BioRad), and washed with 5 column volumes of water (Milli Q, Millipore). HAOS 3-10 fraction obtained from the previous step (15 ml; 1.5 g of oligosaccharides) was applied to this column. The column was eluted with water; 15 ml fraction were collected and analyzed for the presence of HA oligosaccharides. Oligosaccharide containing  
10 fractions eluted before salts (the latter detected with AgNO<sub>3</sub>) were combined and concentrated again on 3 YM Diaflo membrane.

*(d) Analysis of HAOS 3 - 10*

To determine the coupling efficiency of the Sepharose, gel (the same batch) was washed and suspended in water as to prepare a 50 % slurry. From the  
15 suspension of Sepharose-HAOS 3 – 10 conjugate and Sepharose used as a control, 100 µl aliquots were withdrawn in triplicate and added to 2.5 ml of 2.2 N trifluoroacetic acid (TFA, Merck KgaA) in teflon screw capped tube. For hydrolysis, the mixture were flushed with argon and incubated at 100°C for 16 h. At the end of hydrolysis, samples were dried under nitrogen, re-suspended in  
20 water and used for the determination of glucosamine and uronic acid. To determine the extent of uronic acid and glucosamine decomposition for each of the hydrolysis, control samples containing known amounts of UA or GlcNAc were included, and incubated under the same conditions.

Under conditions described above 5, 8, 9, 11 and 15 mg of HAOS 3 – 10 were  
25 coupled per 1 ml of drained Sepharose gel in *two independent experiments*. This results are based on the UA and glucosamine assays.

*(e) Assay used*

The content of the uronic acid in the samples analyzed was determined according to Bitter T. and Muir H. M., *Anal. Biochem.*, 1962, 4, 330 – 334.

30 The hexosamine amounts were analyzed with the method of Rondle C.J.M. and Morgan W.T.J., *Biochem. J.*, 1955, 61, 586 – 593.

Example 9: - Hyaluronic Acid Fragments Sepharose Chromatography (HA-Sepharose Chromatography)

The chromatography matrices containing 8 to 10 mg/ml were prepared as indicated. The enzyme containing sample was dialyzed against 20 mM acetic  
5 buffer + 0.15 M NaCl pH 4.0 and applied to the 25 ml HA-Sepharose column. After washing with the same buffer, the elution was done with the 20 mM acetic buffer with a 0.15 to 1 M gradient of NaCl.

The 1 ml fractions were tested in the hyaluronidase-activity determination test, pooled, dialyzed overnight against 20 mM phosphate buffer pH 6.0 containing  
10 sodium azide and 17 mg/ml trehalose. The protein and activity determination of the pools was carried out. The purification factor of this chromatography step was about 3.

Example 10: - Diol-LiChrospher Chromatography

15 A 20 ml active sample dialyzed against Milli-Q-H<sub>2</sub>O was applied on the Diol-LiChrospher column. The column was then equilibrated with 15 ml Milli-Q-H<sub>2</sub>O and washed 5 min with 2 ml water. The elution of the active sample was done 15 min with 20 mM acetic buffer pH 5.9 (gradient, 0 to 5 mM NaCl) and 35 min with gradient 20 mM to 100 mM acetic acid buffer pH 5.5 containing 5 mM NaCl. The  
20 fractions were assayed for hyaluronidase activity. The active fractions were pooled and dialyzed overnight against 20 mM phosphate buffer pH 6.0 containing sodium azide and 17 mg/ml trehalose. The protein and activity determination of the pools was carried out. The purification factor: 3.

25 Example 11: - RP 18e Chromatography

This purification step can be used only as the last one and is aimed to obtain the sample devoid of salts and other protein impurities (e. g. peptide protease inhibitors). The hyaluronidase activity was completely lost, because manillase is not resistance to organic solvents used in this step. Manillase sample was  
30 applied to the RP 18e column. The 0.25 ml/min fractions were collected. The elution was done in the presence of 0.1% TFA and, gradient water to 99% of acetonitrile was used. The RP-purified samples can be used directly for amino acid sequencing, MALDI measurement, carbohydrate structure analysis and as standard for purification of other batches of manillase.



Example 12: - Activity Determination - Turbidity Reduction Test

The hyaluronidase activity determination was done with the turbidity reduction measurements. Commercially available preparations of hyaluronan (isolated from the different animal tissues and fluids, e.g. human cord, rooster comb) and  
5 hyaluronidases (endo- $\beta$ -glucosaminidases from bovine testes, porcine testes, bee venom; lyases from *Streptomyces hyalurolyticus*) were used for establishing suitable activity assay conditions. The endo- $\beta$ -glucuronidase from *Hirudo medicinalis* was partially purified in our laboratory.

Hyaluronan stock solution (conc. 2 mg/ml) was prepared by dissolving HA in 0.3  
10 M phosphate buffer pH 5.3. This solution was diluted with the same buffer to a concentration of 0.2 mg/ml directly before the test. The enzyme-containing samples were diluted to an appropriate amount of enzyme (0.5 - 5 WHO units) with 20 mM phosphate buffer containing 0.01% of bovine albumin and 77mM of NaCl (enzyme dilution buffer). To 0.1 ml of these samples, 0.1 ml hyaluronan (0.2  
15 mg/ml) solution was added, mixed and incubated 45 minutes at 37°C. The test was done in duplicate. The reaction was stopped by dilution with 1.0 ml of albumin reagent (0.1% of albumin dissolved in 80 mM acetic acid/ 40 mM sodium acetate buffer, pH 3.75). After 10 min incubation at RT or 37°C the optical density at 600 nm was read and the activity was expressed in WHO (IU) units by  
20 comparison (SLT-program) with a standard. The WHO preparation of bovine testicular hyaluronidase (Humphrey J. H., Bull. World Health Org. 1957, 16, 291-294) was used as standard.

Example 13: - Protein Estimation

25 The protein content of column eluents was determined by measuring the ultraviolet absorbance of solutions at 280 nm. The protein concentration of the pooled fractions was determined with the aid of Pierce micromethod. The BSA solution was used as a reference protein.

Example 14: - SDS-PAGE Electrophoresis

30 Electrophoresis was done according to Laemmli procedure (Nature, 1970, 227, 680-685). The following gels were used: 4 to 20% gradient or 12,5% separating gels with 4% stacking gel. Samples were subjected to electrophoresis in the presence of sodium dodecyl sulfate and  $\beta$ -mercaptoethanol. Proteins were

visualized after staining with Coomassie brilliant blue and/or Silver staining (according to Pharmacia instruction).

Example 15: - Isoelectric Focusing

- 5 To pursue isoelectric focusing studies on the manillase preparation, the protocol provided by supplier (Pharmacia) was adopted. Following focusing, the gel was fixed and silver stained (according to Pharmacia protocol).

Example 16: - Preparation of Immunoglobulin from Immune Sera of Rabbits

- 10 (*anti-ConA, anti-hemoglobin and anti-peptide rabbit antibodies*)

The rabbit sera were raised with the use of the following immunogens: concanavalin A lectin, mixture of hemoglobins and peptide-KLH conjugates. The peptide sequence was identical with that of the 14 amino acid N-terminal part of manillase (KEIAVTIDDKNVIA).

- 15 The sera were purified on the Protein A Sepharose (Pharmacia, 17-0780-01) column according to the standard Pharmacia instruction. The purity of the IgG samples were checked with the aid of SDS-PAGE and ELISA-test.

Example 17: - Western-Immunoblot Assay

- 20 Suitable aliquots of the samples and pre-stained protein marker of known molecular weight were subjected to SDS-PAGE as described above. A pre-stained BioRad molecular weight marker was used. The protein was transferred electrophoretically from polyacrylamide gels (0,8 mA/cm<sup>2</sup>) to immobile polyvinylidene difluoride (PVDF) membranes in the presence of transfer buffer for 100
- 25 min. The PVDF membrane was incubated with blocking solution (PBS, pH 7.5 + 2% fat free milk) for 1 h at room temperature. Next, the membrane was incubated 2 h at room temperature with the antibody, appropriately diluted with the blocking solution. The membrane was washed with TBS+0.05% Tween 20, pH 7.5 and incubated for 2 h at room temperature with (a second antibody) goat anti-rabbit-
- 30 alkaline phosphate conjugate, BioRad. The membrane was washed two times with TBS+Tween 20 and incubated 10 min with BCIP alkaline phosphatase substrate solution. Adding a stopping buffer terminated the reaction.

Example 18: - Amino Acid Sequencing

The sequence of N-terminal 33 amino acid residues of the manillase was obtained by Edman degradation. After SDS-PAGE of manillase-active samples, the bands were transferred onto PDVF membrane, stained with Coomassie Blue, cut-out and sequenced. The same amino sequence was found for the sample obtained after the last purification step with the aid of RP-column chromatography.

Example 19: - pH Dependence of Enzyme Activity

(for hyaluronidase isolated from *Hirudinaria manillensis* and *Hirudo medicinalis* leech heads)

Samples of hyaluronidase used in this experiment were extracted either from *Hirudinaria manillensis* or *Hirudo medicinalis* leech heads and partially purified with the aid of ammonium sulfate precipitation and cation exchange chromatography. Each sample containing 500 WHO units/ml was incubated at -20°C, +4°C and 37°C at a range of pHs from 2.6 to 9.0 (20 mM acetic for pH 2.6 to 5; 20 mM phosphate buffer for pH 5 to 9). The enzyme activity was measured after 1, 2 and 7 days incubation periods. At both acid and alkaline extremes of pH, inhibition of activity to the same extent was observed for both hyaluronidases. However, during longer incubation periods manillase was more stable than *Hirudo medicinalis* hyaluronidase: e.g. after 7 days incubation at pH 7.0 at +4°C and 37°C - manillase retained 75% and 60% of the starting activity, respectively. The *Hirudo medicinalis* hyaluronidase incubated at the same conditions was already inactive after 1 day.

25

Example 20: - Stability Measurement of Hyaluronidases in the Presence of Dog's Serum (for hyaluronidase isolated from *Hirudinaria manillensis* and *Hirudo medicinalis* leech heads)

The 5 kU/ml samples of manillase, *Hirudo medicinalis* and bovine testes hyaluronidase were diluted with dog's or rat's citrated plasma to a final concentration of 250 U/ml. Next, these solutions were incubated at -20°C, +4°C and +37°C for 0 to 7 days. The controls containing the same hyaluronidases, diluted in buffer were included in this experiment. Finally, the hyaluronidase activity was measured.

Example 21: - Contaminating Enzyme Activities

At each stage of the purification procedure for leech hyaluronidase, the preparation was checked for other enzymes capable of degrading protein with the aid of universal protease substrate (Boehringer Mannheim, cat. no. 1080 733) according to Twining S. S. (Anal. Biochem., 1984, 143, 30-34).

Example 22: - Influence of Heparin on Hyaluronidase Activity

Cleavage of a hyaluronan by hyaluronidases results in the liberation of reducing sugars. The amount of the liberated sugars was measured colorimetrically by the modified method of Park (Park J. & Johnson M.; J. Biol. Chem. 1949, 181, 149). For the measurement of the influence of heparin on the activity of manillase and bovine testes hyaluronidase, two activity determination were carried out: one in the presence of heparin, and second without heparin. Hyaluronidase samples, 25  $\mu$ l (3.2 WHO units) were incubated 30 min at 37°C with 25  $\mu$ l of the heparin (Liquemin, Fa. Hoffmann LaRoche) solution, containing 0 to 24 units of heparin. Then, 50  $\mu$ l of hyaluronan (2.5 mg/ml) was added and the incubation was continued for 30 min at 37°C. The reaction was terminated by heating for 2 min at 100°C. Next, 100  $\mu$ l of carbonate-cyanide solution and 100  $\mu$ l of potassium ferricyanide solution were added to the inactivated digest. The samples were heated in a boiling water bath for 15 min and then cooled in an ice bath. Afterwards, 0.75  $\mu$ l of ferric ammonium sulfate solution was added to the reaction mixtures. After 15 min incubation at RT, the color developed was measured at 690 nm in a Shimadzu spectrophotometer. Suitable blanks and no-enzyme controls were included in each assay. The expected reducing sugar (glucuronic acid or N-acetyl-glucosamine, 1 to 15  $\mu$ g) for the type of sample under analysis was used as standard.

Example 23: - Deglycosylation of the Manillase

The samples of manillase were deglycosylated with the aid of PNGase F enzyme (BioLabs Art. No. 701 L) according to supplier instruction. The deglycosylation was done under denaturing and native conditions. The O-glycanase, neuraminidase and neuraminidase + O-glycanase treatments were done according to Boehringer Mannheim standard prescriptions. All samples were characterized with the SDS-PAGE and activity determination test.

**Example 24:** - Construction of the *E. coli* Expression Vector (Fig. 11)

For expression in *E. coli* we used a modified version of the plasmid pASK75, which carries the tet promoter region. {Skerra, Gene 151, (1994), pp 131-135 }.

- 5 The modification we made by cloning a new linker between the XbaI and Hind III sites. The new linker contains the ompA leader sequence, another multiple cloning site and a 6xHis-tag instead of the strep-tag.

Linker sequence which was cloned in pASK75.

```

Xba I
119 CTAGATAACG AGGGCAAAAA ATGAAAAAGA CAGCTATCGC GATTGCAGTG GCACTGGCTG
    TATTGC TCCCGTTTTT TACTTTTTCT GTCGATAGCG CTAACGTAC CGTGACCGAC
                1MetLysLysT hrAlaIleAlaIleAlaValAlaLeuAlaG
                  ClaI      EcoRI      SstI      KpnI      SmaI      BamHI
179 GTTTCGCTAC CGTAGCGCAG GC AT CGA TGA ATT CGA GCT CGG TAC CCG GGG
    CAAAGCGATG GCATCGCGTC CG TA GCT ACT TAA GCT CGA GCC ATG GGC CCC
141 yPheAlaThrValAlaGlnAla
    XhoI      SstI      PstI      Eco47III
230 ATC CCT CGA GGT CGA CCT GCA GGC AGC GCTATGAGAGGATCGCATCACCATCACCA
    TAG GGA GCT CCA GCT GGA GGT CCG TCG CGATACTCTCCTAGCGTAGTGGTAGTGGT
                Hind III      1AlaMetArgGlySerHisHisHisHisHis
286 TCACTAATAGA
    AGTGATTATCTTGA
101 sHis.....

```

- 10 To construct the expression vector for manillase it was necessary to introduce 5' Cla I and 3' Eco47III restriction sites by PCR method. Therefore the two primers
- 5' ATC GAT AAA GAG ATT GCC GTG AC and
- 3' GTT GTT TCC GAT GCT AAA GCG CT

were used. The PCR product first was cloned into the PCR II vector system

- 15 (Invitrogen) and sequenced.

In a second step the manillase gene was cloned into the modified pASK75 vector using the restriction sites 5' ClaI and 3' Eco47III.

After expressing and proving the activity of this recombinant manillase in a second PCR reaction the His-tag was removed and the start codon of the

- 20 manillase gene was directly fused to the omp A leader sequence. The primers for this PCR reaction were:

5' ACC GTA GCG CAG GCC AAA GAG ATT GCC GTG and

3' CAC GGC AAT CTC TTT GGC CTG CGC TAC GGT.

- 25 **Example 25:** - Construction of the Baculo Donor Plasmid (Fig. 12)

For expression of manillase in the Baculo virus expression system the Bac-To-Bac™ Baculovirus Expression System from Gibco Life Technologies was used.

To get a section system the Honeybee melitin leader sequence was fused to the

manillase gene and to introduce the restriction sites 5' BamHI and 3' KpnI one single PCR reaction was carried out.

5' Primer:

CGG ATC CAT GAA ATT CTT AGT CAA CGT TGC CCT TGT TTT TAT GGT

5 CGT ATA CAT TTC TTA CAT CTA TGC GAA AGA GAT TGC CGT GAC

3' Primer:

AAT GTT GAA GCA TAA GGT ACC

The PCR product was cloned into the PCR II Vector (Invitrogen) and sequenced.

Then the Melitin – Manillase Fusion was cloned into the pFastBac vector using  
10 the restriction sites 5' BamHI and 3' KpnI (Fig. 12).

Example 26: - Construction of the Yeast Expression Vector (Fig. 13)

For expression in yeast we used the pichia multi copy expression system (Invitrogen). To construct the expression vector for manillase we used the PCR  
15 amplification method of the manillase gene in such a way that compatible restriction ends (5' EcoR I, 3' Not I) are generated for ligation into the appropriate vector (pPIC9K). Therefore the following primers were used:

5' GTA GAA TTC AAA GAG ATT GCC GTG ACA

3' GAT GCT AAT GTT GAA GCA TAA TGA GCG GCC GC

20 Before transforming the Pichia Spheroplasts the expression vector has to be linearized with Sal I.

Example 26: - Expression in E. coli

In the expression vector pRG72, which contains the structural gene of Sarastatin  
25 fused to the ompA leader sequence, was transformed into W3110 competent cells. The cells were grown to a mid-log phase, and the promoter was then induced by adding 200µg aTC / l. 1 h thereafter the recombinant manillase could be clearly detected.

30 Example 27: - Generation of Recombinant Baculoviruses and Manillase  
Expression with the Bac-To-Bac Expression System

The donor plasmid pTD13 was transformed into DH10Bac competent cells which contain the bacmid with a mini-attTn7 target site and the helper plasmid. The mini-Tn7 element on the donor plasmid can transpose to the a mini-attTn7 target

- site on the bacmid in the presence of transposition proteins provided by the helper plasmid. Colonies containing recombinant bacmids were identified by disruption of the *lacZ* gene. High molecular weight mini-prep DNA prepared from selected *E. coli* clones containing the recombinant bacmid, and this DNA was
- 5 then used to transfect insect cells.
- Detailed description could be find in the instruction manual of the expression kit.

Example 28: - Expression in yeast

- To be sure to have integrated the manillase gene the colonies have to be
- 10 screened for His<sup>+</sup> Mut<sup>+</sup>-mutants
- Using a single colony, inoculate 100 ml Medium i a 1 l flask. Growing conditions are: 28 – 30°C, 250 rpm, up to OD 2-6. To induce expression, first cetrifuge the culture, decant to supernatant and re-suspend the cell pellet in new medium using 1/5 of the original culture volume. Add 100% methanol to a final
- 15 concentration of 0,5% every 24 hours to maintain induction. After max 6 days supernatant is analyzed by SDS-Page and the activity assay.

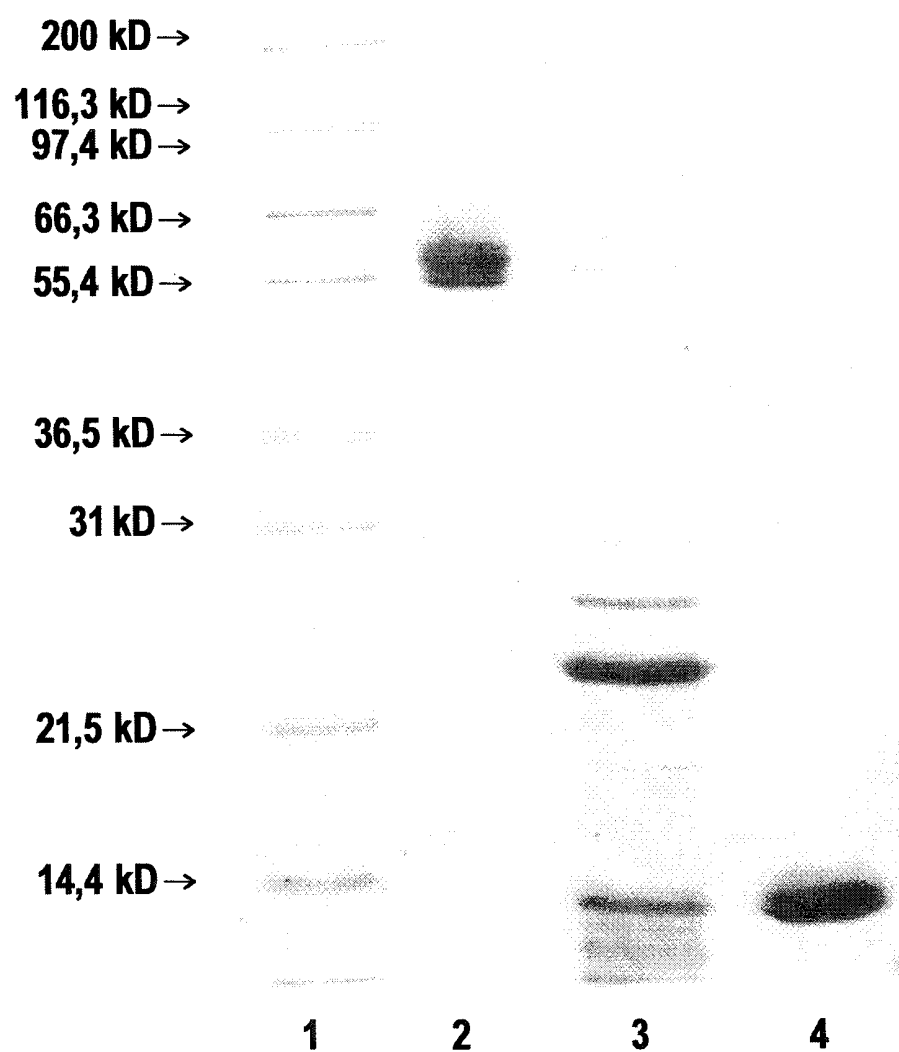
Patent Claims

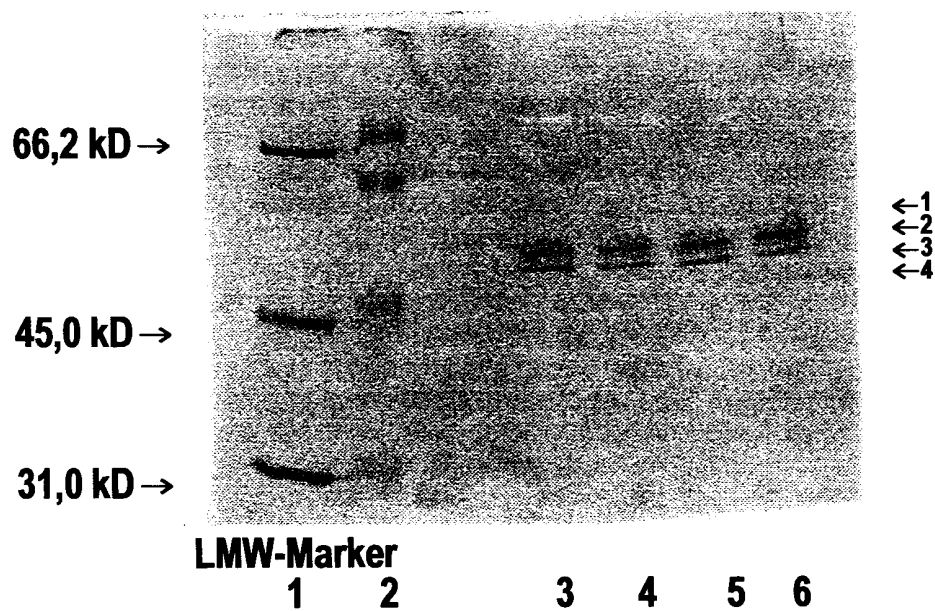
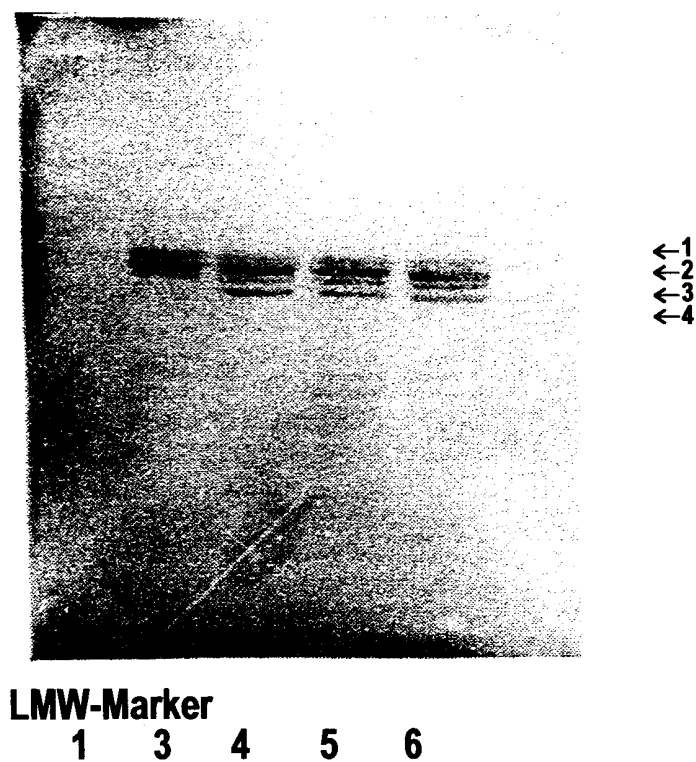
1. A purified protein isolated from the leech species *Hirudinaria manillensis* having the biological activity of a hyaluronidase which is not influenced in its activity by heparin, characterized in that it has a molecular weight of 53 – 60 dependent on glycosylation.
2. A glycosylated protein according to claim 1 having a molecular weight of 58 (  $\pm 2$ ).
3. A non-glycosylated protein according to claim 1 having a molecular weight of 54 (  $\pm 2$ ).
4. A protein according to any of claims 1 – 3 having an isoelectric point of 7.2 – 8.0.
5. A protein according to any of claims 1 – 4 having the amino acid sequence given in Fig. 7 and SEQ ID No. 1.
6. A protein according to claims 1 – 5 having a specific enzymatic activity of > 100 kU / mg protein.
7. A process for isolating and purifying the protein as defined in claims 1 – 6 comprising the following steps
  - (i) homogenization of heads of leeches of the species *Hirudinaria manillensis* with an acid buffer and centrifugation,
  - (ii) ammonium sulfate precipitation of the supernatant of step (i),
  - (iii) cation exchange chromatography,
  - (iv) concanavalin A affinity chromatography
  - (v) hydrophobic interaction chromatography
  - (vi) affinity chromatography on matrices coated with hyaluronic acid fragments
  - (vii) gel permeation chromatography, and optionally
  - (viii) enzymatic or chemical de-glycosylation of the purified protein.



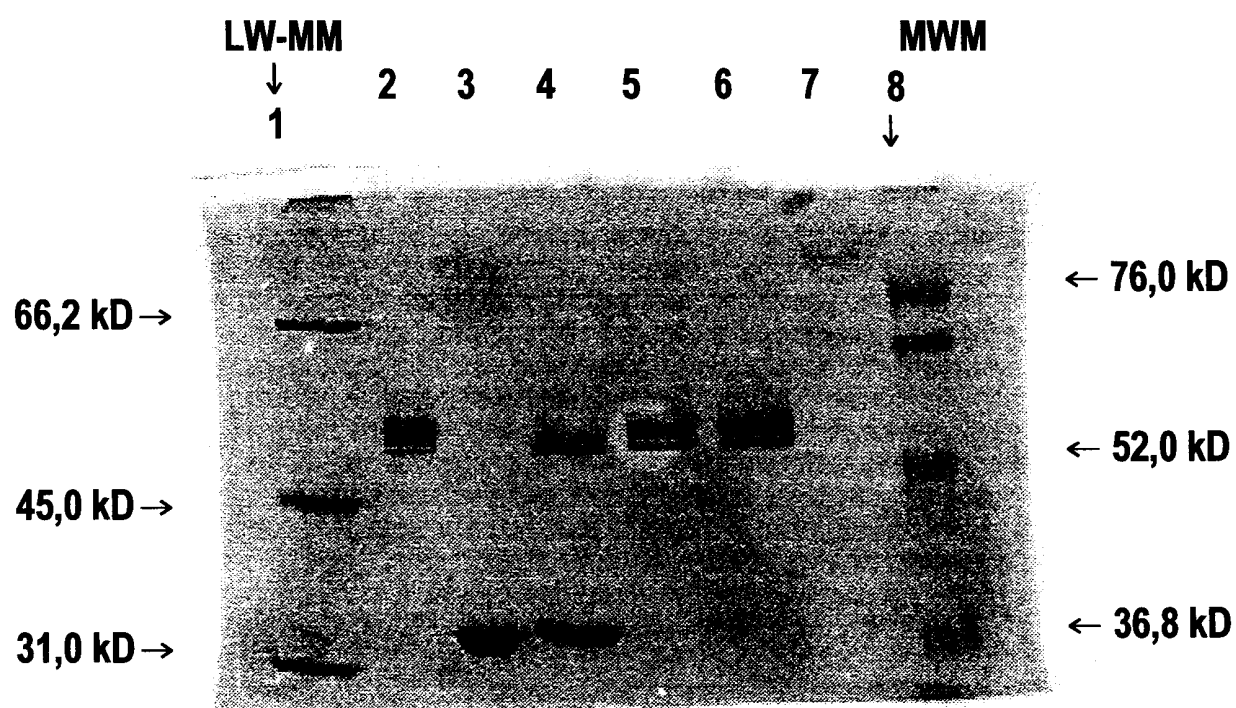
8. A protein having the biological activity of a hyaluronidase which is not influenced in its activity by heparin and having a molecular weight of 53 – 60 dependent on glycosylation, obtainable by the process steps of claim 7.
9. A protein according to claim 8 having a specific enzymatic activity of > 100 kU / mg protein.
10. A DNA sequence coding for a protein of claim 1 and 9.
11. A DNA sequence coding for a protein of claim 8 comprising any nucleotide sequence depicted in Fig. 8 (SEQ. ID No. 2) , Fig. 9 (SEQ. ID No. 4) and Fig.10 (SEQ ID No. 6).
12. A recombinant protein having the biological activity of a hyaluronidase encoded by any a DNA sequence of claim 11.
13. A recombinant protein with the biological activity of a hyaluronidase and a molecular weight of 55 – 59 dependent on glycosylation having any amino acid sequence depicted in Fig. 8, 9 and 10 (SEQ. ID Nos. 3, 5, 7) or a sequence which has a homology to said sequences of at least 80%.
14. An expression vector comprising a DNA sequence of claim 10 or 11.
15. A host cell suitable for the expression of a protein of claim 12 or 13 which was transformed with a vector of claim 14.
16. A protein according to any of claims 1 – 6, 8, 9, 12 and 13 as a medicament.
17. A pharmaceutical composition comprising the protein of claim 16 and a pharmaceutically acceptable diluent, carrier or excipient therefor.

18. A pharmaceutical composition comprising additionally a pharmacologically active compound.
- 5 19. A pharmaceutical composition according to claim 18, wherein the pharmacological active compound is heparin.
20. The use of a protein according to any of claims 1 – 6, 8, 9, 12 and 13 in the manufacture of a medicament for treating myocardial, cardiovascular and thrombotic disorders and tumors.

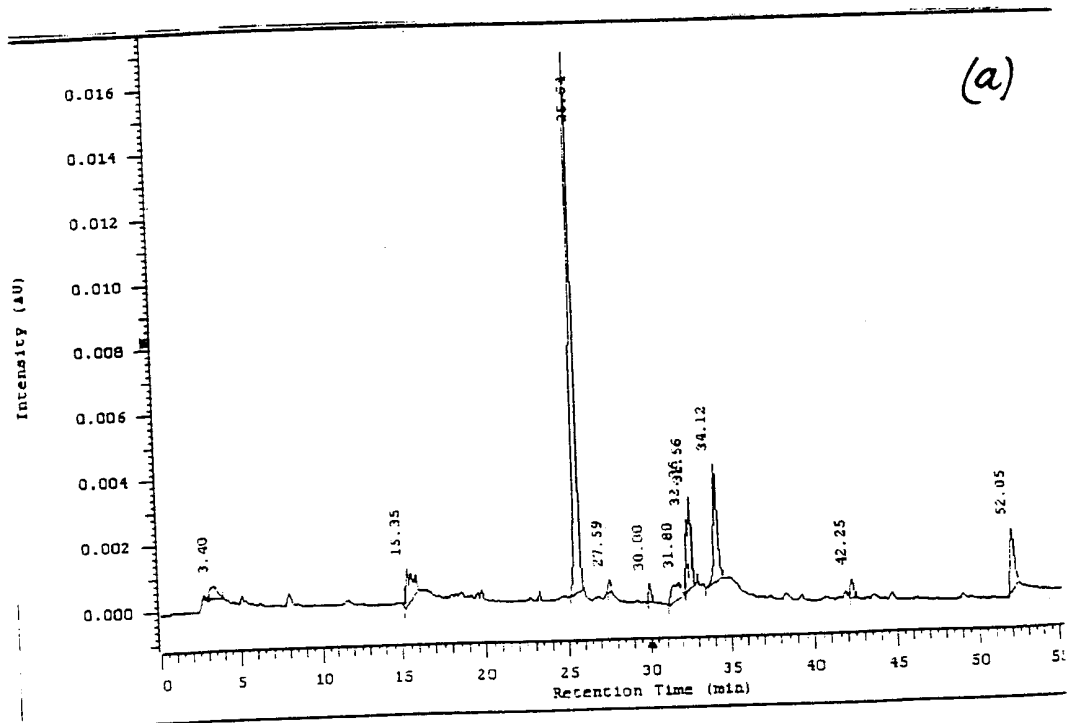
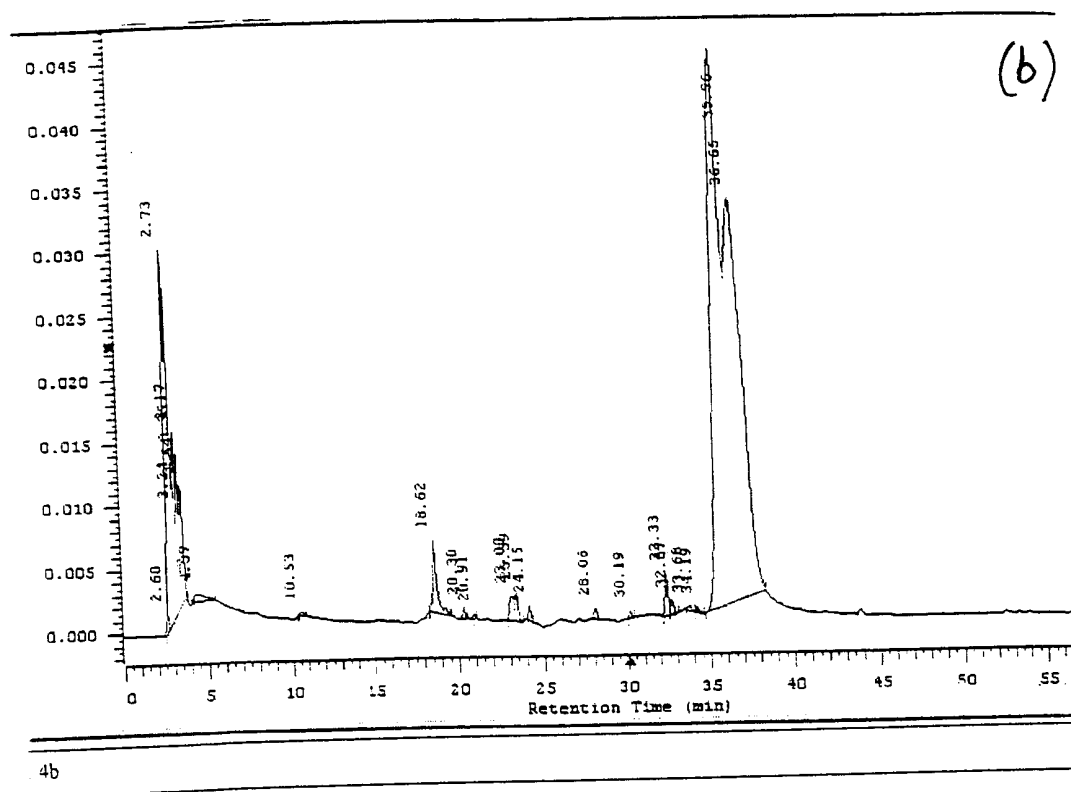
**Fig.1**

**Fig.2****a) - SDS-PAGE****b) - SDS-PAGE-Western blot**

**Fig.3**



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**Fig. 4**

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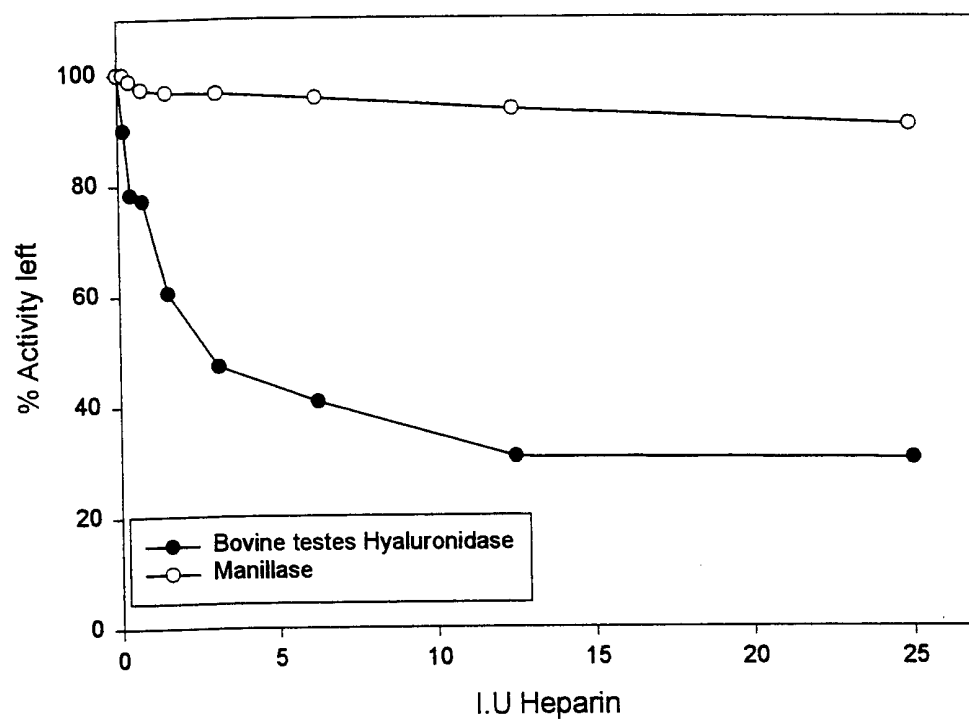
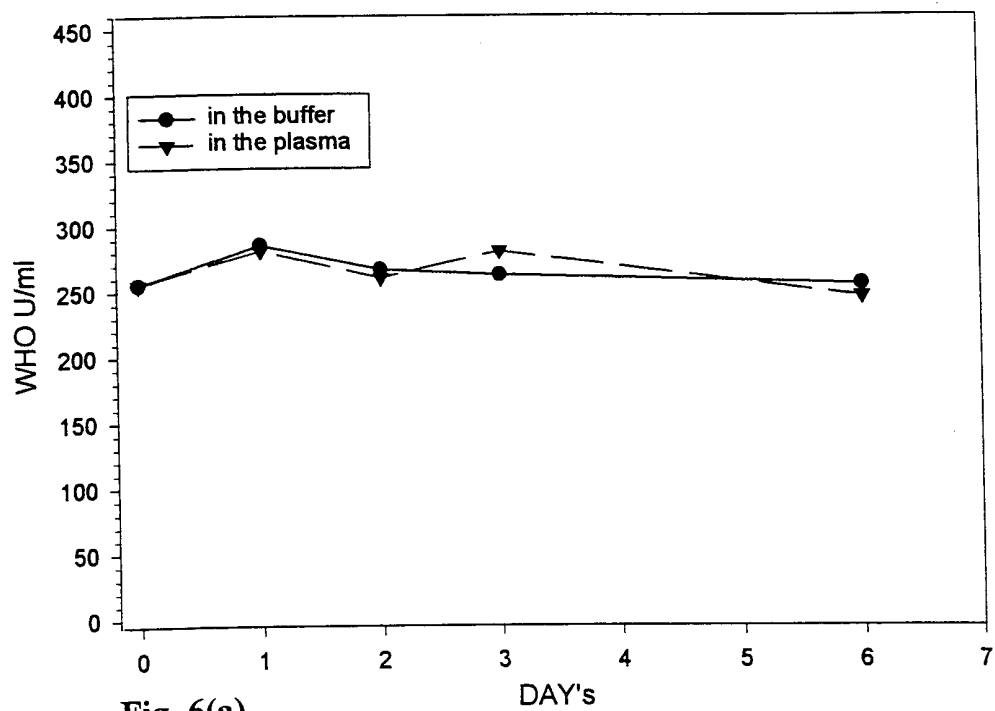


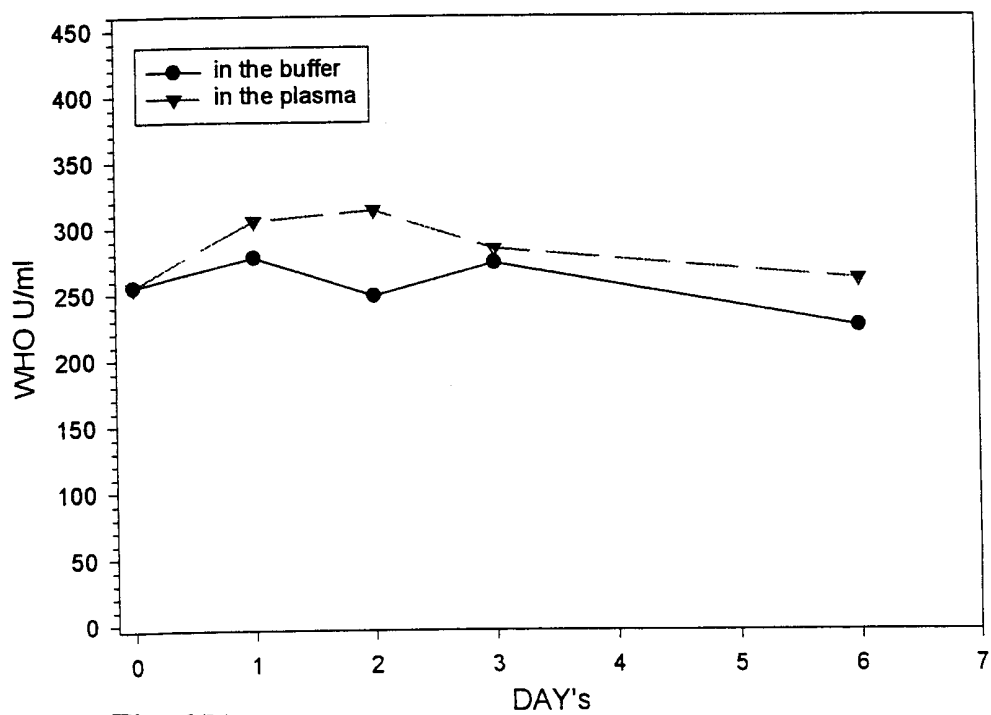
Fig.5

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activity of Manillase at 4°C

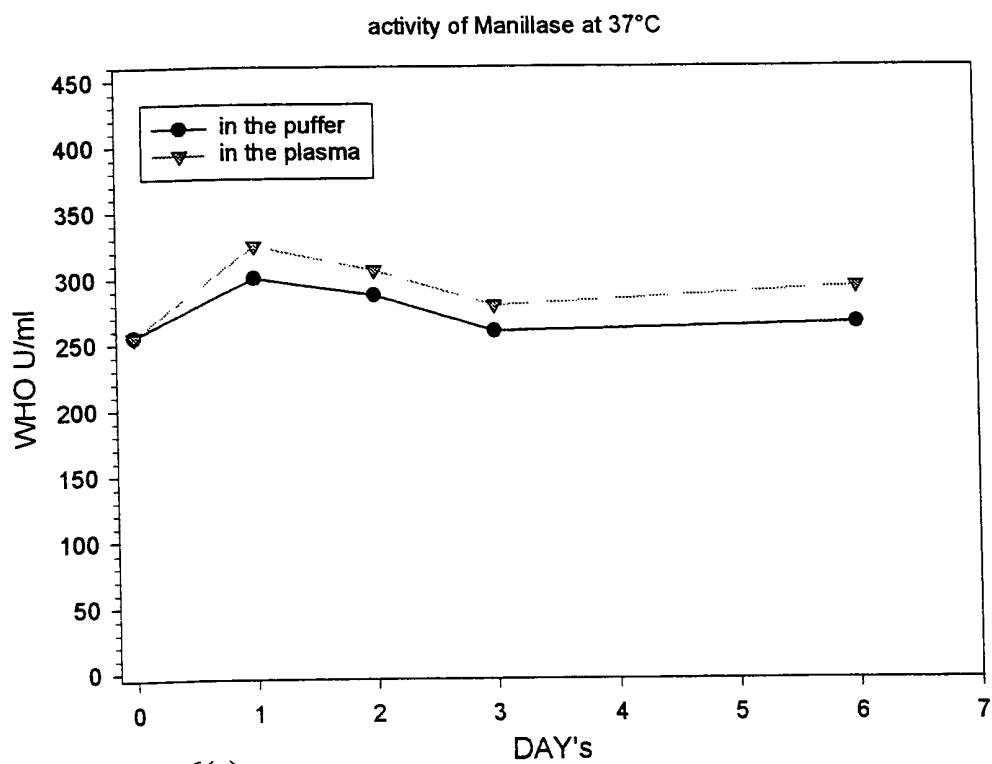
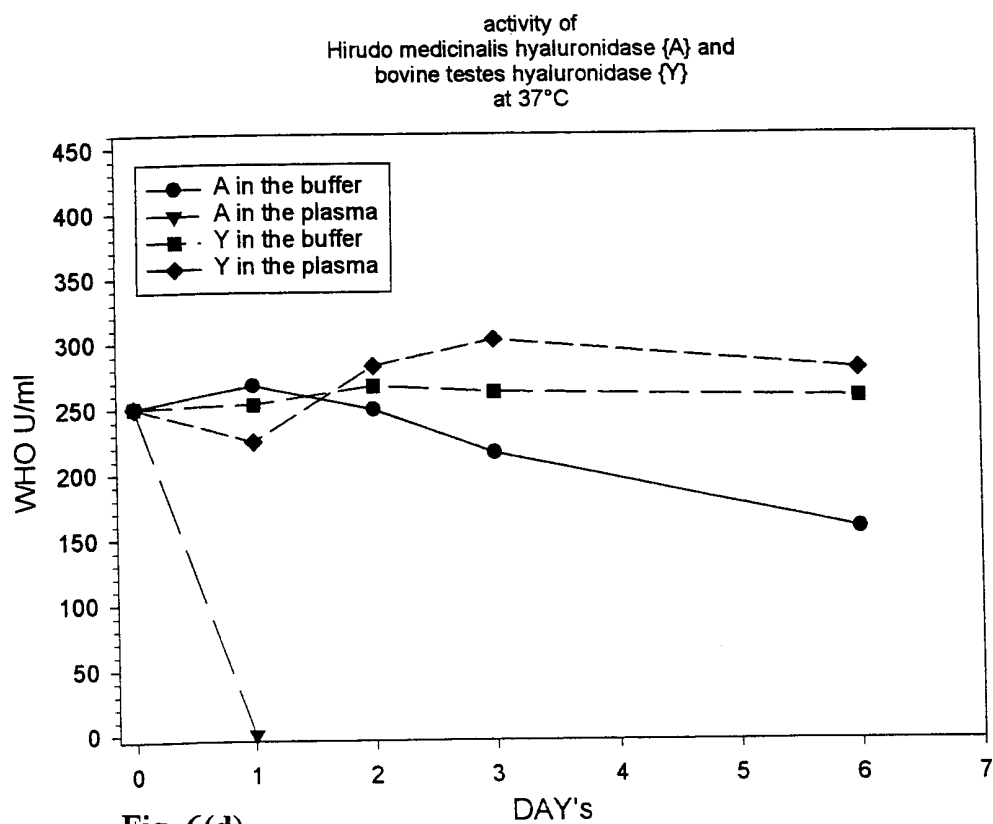
**Fig. 6(a)**

activity of Manillase at -20°C

**Fig. 6(b)**



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**Fig. 6(c)****Fig. 6(d)**

**Fig. 7**

KEIAVTIDDK	NVIASVSESF	HGVAFDASLF	SPKGLWSFVD	ITSPKLFKLL	50
EGLSPGYFRV	GGTFANWLFF	DLDENNKWKD	YWAFKDKTPE	TATITRRWLF	100
RKQNNLKKET	EDDLVKLTKG	SKMRLFLDLN	AEVRTGYEIG	KKMTSTWDSS	150
EAEKLFKYCV	SKGYGDNIDW	ELGNEPDHTS	AHNLTEKQVG	EDFKALHKVL	200
EKYPTLNKGS	LVGPDVGWMG	VSIVKGLADG	AGDLVTAFTL	HQYYFDGNTS	250
DVSTYLDATY	FKKLQQLFDK	VKDVLKNSQH	KDKPLWLGET	SSGYNSGTKD	300
VSDRYVSGFL	TLDKLGLSAA	NNVKVVIRQT	IYNGYYGLLD	KNTLEPNPDY	350
WLMHVVHNSLV	GNTVFKVDVS	DPTNKARVYA	QCTKTNSKHT	QSRYYKGSIT	400
IFALNVGDED	VTLKIDQYGG	KKIYSYILTP	EGGQLTSQKV	LLNGKELKLV	450
SDQLPELNAN	ESKTSFTLSP	KTFGFFVSD	ANVEACKK		488

**Fig. 8:**

AAA K	GAG E	ATT I	GCC A	GTG V	ACA T	ATT I	GAC D	GAT D	AAG K	AAT N	GTG V
ATT I	GCA A	TCT S	GCC A	AGT S	GGG G	TCT S	TTC F	CTT L	GGA G	GTT V	GCC A
TTT F	GAT D	GCG A	TCT S	CTA L	TTT F	TCG S	CCC P	AAG K	GGT G	CTT L	TGG W
AGC S	TTT F	GTT V	GAT D	ATT I	ACC T	TCT S	CCA P	AAA K	TTG L	TTC F	AAA K
TTG L	CTG L	GAA E	GGA G	CTT L	TCT S	CCT P	GGA G	TAC Y	TTC F	AGG R	GTT V
GGC G	GGA G	ACG T	TTT F	GCC A	AAT N	TGG W	CTG L	TTT F	TTT F	GAC D	TTG L
GAC D	GAA E	AAT N	AAT N	AAG K	TGG W	AAG K	GAT D	TAT Y	TGG W	GCT A	TTT F
AAA K	GAC D	AAA K	ACC T	CCC P	GAA E	ACT T	GCG A	ACA T	ATA I	ACA T	AGG R
AGA R	TGG W	CTG L	TTC F	AGA R	AAA K	CAA Q	AAT N	AAT N	CTG L	AAA K	AAG K
GAG E	ACT T	TTT F	GAC D	AAT N	TTA L	GTG V	AAA K	CTA L	ACA T	AAG K	GGA G
AGC S	AAG K	ATG M	AGA R	TTG L	TTA L	TTC F	GAT D	TTG L	AAT N	GCC A	GAA E
GTG V	AGG R	ACT T	GGT G	TAT Y	GAA E	ATT I	GGA G	AAG K	AAG K	ATG M	ACA T
TCC S	ACT T	TGG W	GAT D	TCA S	TCG S	GAG E	GCT A	GAA E	AAG K	TTA L	TTT F
AAA K	TAT Y	TGT C	GTG V	TCA S	AAA K	GGT G	TAC Y	GGA G	GAC D	AAT N	ATC I
GAT D	TGG W	GAA E	CTT L	GGA G	AAT N	GAA E	CCG P	GAC D	CAC H	ACC T	TCA S
GCT A	CAC H	AAT N	TTA L	ACT T	GAA E	AAG K	CAG Q	GTT V	GGA G	GAA E	GAT D
TTT F	AAA K	GCA A	CTG L	CAT H	AAA K	GTT V	CTA L	GAG E	AAA K	TAT Y	CCA P

**Fig 8 (contnd)**

ACT T	CTT L	AAC N	AAG K	GGA G	TCG S	CTC L	GTT V	GGT G	CCA P	GAT D	GTA V
GGG G	TGG W	ATG M	GGC G	GTC V	AGT S	WAC Y	GTC V	AAG K	GGA G	TTG L	GCA A
GAC D	GAG E	GCR A	GGT G	GAC D	CAT H	GTA V	ACK T	GCT A	TTT F	ACA T	CTC L
CAC H	CAA Q	TAT Y	TAT Y	TTC F	GAT D	GGA G	AAC N	ACY T	TCT S	GAT D	GTA V
TCA S	ATA I	TAT Y	CTT L	GAT D	GCC A	ACA T	TAC Y	TTT F	AAG K	AAG K	CTG L
CAA Q	CAA Q	CTA L	TTT F	GAT D	AAA K	GTG V	AAA K	GAT D	GTT V	TTG L	AAA K
GAT D	TCT S	CCA P	CAT H	AAA K	GAC D	GAA E	CCA P	TTA L	TGG W	CTT L	GGA G
GAA E	ACA T	AGT S	TCT S	GGA G	TAC Y	AAC N	AGC S	GGC G	ACA T	GAA E	GAT D
GTA V	TCC S	GAT D	CGA R	TAT Y	GTT V	TCA S	GGA G	TTT F	CTA L	ACA T	TTA L
GAC D	AAG K	TTG L	GGT G	CTC L	AGT S	GCA A	GCC A	AAC N	AAT N	GTA V	AAG K
GTT V	GTT V	ATA I	AGA R	CAG Q	ACA T	ATA I	TAC Y	AAT N	GGA G	TAT Y	TAT Y
GGT G	CTC L	CTT L	GAC D	AAA K	AAC N	ACT T	TTA L	GAG E	CCG P	AAT N	CCG P
GAT D	TAC Y	TGG W	TTA L	ATG M	CAT H	GTT V	CAT H	AAT N	TCT S	TTG L	GTC V
GGA G	AAT N	ACA T	GTT V	TTT F	AAA K	GTT V	GAC D	GTT V	AGT S	GAT D	CCA P
ACT T	AAT N	AAA K	GCA A	AGA R	GTT V	TAC Y	GCG A	CAA Q	TGT C	ACC T	AAA K
ACA T	AAT N	AGC S	AAA K	CAT H	ACT T	CAA Q	AGC S	AGA R	TAT Y	TAC Y	AAG K
GGC G	TCT S	TTG L	ACA T	ATC I	TTT F	GCA A	CTT L	AAT N	GTT V	GGA G	GAT D

**Fig 8 (contnd)**

GGA G	GAT D	GTA V	ACG T	TTA L	AAG K	ATC I	GGT G	CAA Q	TAC Y	AGC S	GGT G
AAA K	AAA K	ATT I	TAT Y	TCA S	TAC Y	ATT I	CTG L	ACA T	CCT P	GAA E	GGA G
GGA G	CAA Q	CTT L	ACA T	TCA S	CAG Q	AAA K	GTT V	CTC L	TTG L	AAT N	GGA G
AAG K	GAA E	TTG L	AAC N	TTA L	GTG V	TCT S	GAT D	CAG Q	TTA L	CCA P	GAA E
CTA L	AAT N	GCA A	GAT D	GAA E	TCC S	AAA K	ACA T	TCT S	TTC F	ACC T	TTA L
TCC S	CCA P	AAG K	ACA T	TTT F	GGT G	TTT F	TTT F	GTT V	GTT V	TCC S	GAT D
GCT A	AAT N	GTT V	GAA E	GCA A	TGY C	AAR K	AAR K				

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**Fig. 9:**

AAA K	GAG E	ATT I	GCC A	GTG V	ACA T	ATT I	GAC D	GAT D	AAG K	AAT N	GTG V
ATT I	GCA A	TCT S	GCC A	AGT S	GAG E	TCT S	TTC F	CAT H	GGA G	GTT V	GCC A
TTT F	GAT D	GCG A	TCT S	CTA L	TTT F	TCG S	CCC P	AAG K	GGT G	CTT L	TGG W
AGC S	TTT F	GTT V	GAT D	ATT I	ACC T	TCT S	CCA P	AAA K	TTG L	TTC F	AAA K
TTG L	CTG L	GAA E	GGA G	CTT L	TCT S	CCT P	GGA G	TAC Y	TTC F	AGG R	GTT V
GGC G	GGA G	ACG T	TTT F	GCC A	AAT N	CGG R	CTG L	TTT F	TTT F	GAC D	TTG L
GAC D	GAA E	AAT N	AAT N	AAG K	TGG W	AAR K	GAT D	TAT Y	TGG W	GCT A	TTT F
AAA K	GAC D	AAA K	ACC T	CCC P	GAA E	ACT T	GCG A	ACA T	ATA I	ACA T	AGG R
AGA R	TGG W	CTG L	TTC F	AGA R	AAA K	CAA Q	AAT N	AAT N	CTG L	AAA K	AAG K
GAG E	ACT T	TTT F	GAC D	AAT N	TTA L	GTG V	AAA K	CTA L	ACA T	AAG K	GGA G
AGC S	AAG K	ATG M	AGA R	TTG L	TTA L	TTC F	GAT D	TTG L	AAT N	GCC A	GAA E
GTG V	AGG R	ACT T	GGT G	TAT Y	GAA E	ATT I	GGA G	AAG K	AAG K	ATG M	ACA T
TCC S	ACT T	TGG W	GAT D	TCA S	TCG S	GAG E	GCT A	GAA E	AAG K	TTA L	TTT F
AAA K	TAT Y	TGT C	GTG V	TCA S	AAA K	GGT G	TAC Y	GGA G	GAC D	AAT N	ATC I
GAT D	TGG W	GAA E	CTT L	GGG G	AAT N	GGA G	CCG P	GAC D	CAC H	ACC T	TCA S
GCT A	CAC H	AAT N	TTA L	ACT T	GAA E	AAG K	CAG Q	GTT V	GGA G	GAA E	GAT D
TTT F	AAA K	GCA A	CTG L	CAT H	AAA K	GTT V	CTA L	GAG E	AAA K	TAT Y	CCA P
ACT T	CTT L	AAC N	AAG K	GGA G	TCG S	CTC L	GTT V	GGT G	CCA P	GAT D	GTA V

**Fig 9 (contnd)**

GGG G	TGG W	ATG M	GGC G	GTC V	AGT S	TAC Y	GTC V	AAG K	GGA G	TTG L	GCA A
GAC D	GAG E	GCA A	GGT G	GAC D	CAT H	GTA V	ACT T	GCT A	TTT F	ACA T	CTC L
CAC H	CAA Q	TAT Y	TAT Y	TTC F	GAT D	GGA G	AAC N	ACC T	TCT S	GAT D	GTA V
TCA S	ATA I	TAT Y	CTT L	GAT D	GCC A	ACA T	TAC Y	TTT F	AAG K	AAG K	CTG L
CAA Q	CAA Q	CTA L	TTT F	GAT D	AAA K	GTG V	AAA K	GAT D	GTT V	TTG L	AAA K
GAT D	TCT S	CCA P	CAT H	AAA K	GAC D	AAA K	CCA P	TTA L	TGG W	CTT L	GGA G
GAA E	ACA T	AGT S	TCT S	GGA G	TAC Y	AAC N	AGC S	GGC G	ACA T	GAA E	GAT D
GTA V	TCC S	GAT D	CGA R	TAT Y	GTT V	TCA S	GGA G	TTT F	CTA L	ACA T	TTA L
GAC D	AAG K	TTG L	GGT G	CTC L	AGT S	GCA A	GCC A	AAC N	AAT N	GTA V	AAG K
GTT V	GTT V	ATA I	AGA R	CAG Q	ACA T	ATA I	TAC Y	AGT S	GGA G	TAT Y	TAT Y
GGT G	CCC P	CTT L	GAC D	AAA K	AAC N	ACT T	TTA L	GAG E	CCA P	AAT N	CCG P
GAT D	TAC Y	TGG W	TTA L	ATG M	CAT H	GTT V	CAT H	AAT N	TCT S	TTG L	GTC V
GGA G	AAT N	ACA T	GTT V	TTT F	AAA K	GTT V	GAC D	GTT V	AGT S	GAT D	CCA P
ACT T	AAT N	AAA K	GCA A	AGA R	GTT V	TAC Y	GCG A	CAA Q	TGT C	ACC T	AAA K
ACA T	AAT N	AGC S	AAA K	CAT H	ACT T	CAA Q	AGC S	AGA R	TAT Y	TAC Y	AAG K
GGC G	TCT S	TTG L	ACA T	ATC I	TTT F	GCA A	CTT L	AAT N	GTT V	GGA G	GAT D
GAA E	GAT D	GTA V	ACG T	TTA L	AAG K	ATC I	GGT G	CAA Q	TAC Y	AGC S	GGT G

**Fig 9 (contnd)**

AAA K	AAA K	ATT I	TAT Y	TCA S	TAC Y	ATT I	CTG L	ACA T	CCT P	GAA E	GGA G
GGA G	CAA Q	CTT L	ACA T	TCA S	CAG Q	AAA K	GTT V	CTC L	TTG L	AAT N	GGA G
AAG K	GAA E	TTG L	AAC N	TTA L	RTG V	TCT S	GAT D	CAG Q	TTA L	CCA P	CAA Q
CTA L	AAT N	GCA A	YAT D	GAA E	TCC S	AAA K	ACA T	TCT S	TTC F	ACC T	TTA L
TCC S	CCA P	AAG K	ACA T	TTT F	GGT G	TTT F	TTT F	GTT V	GTT V	TCC S	GAT D
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**Fig. 10:**

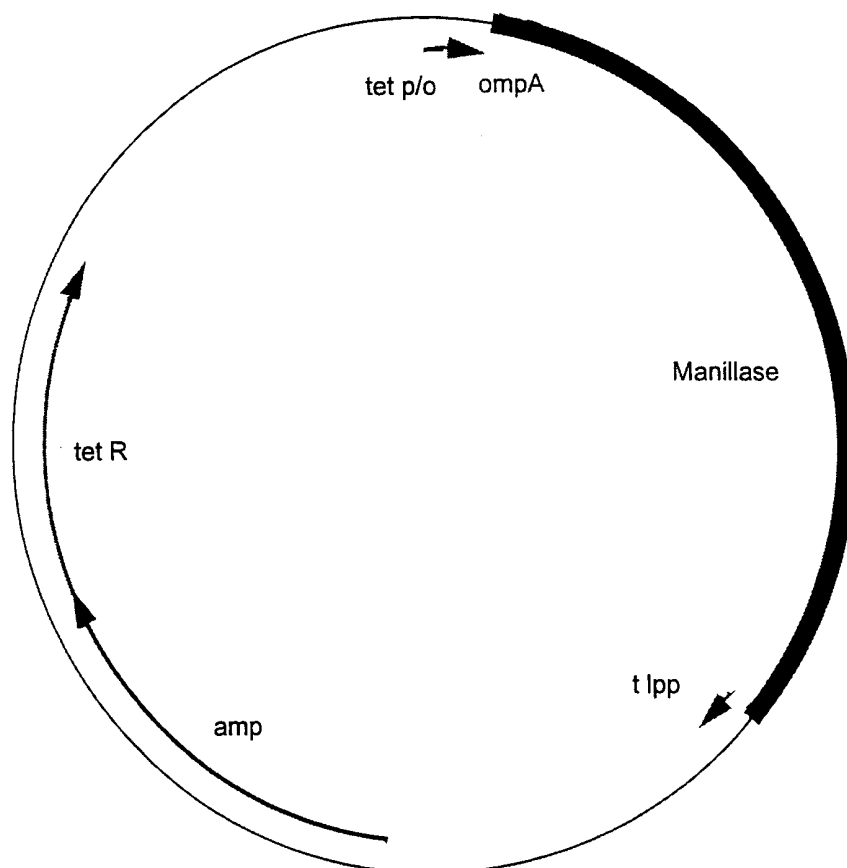
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TTT F	GAT D	GCG A	TCT S	CTA L	TTC F	TCG S	CCC P	AAG K	GGT G	CCT P	TGG W
AGC S	TTT F	GTT V	AAT N	ATT I	ACC T	TCT S	CCA P	AAA K	TTG L	TTC F	AAA K
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GAC D	GAA E	AAT N	AAT N	AAG K	TGG W	AAG K	GAT D	TAT Y	TGG W	GCT A	TTT F
AAA K	GAC D	AAA K	ACC T	CCC P	GAA E	ACT T	GCG A	ACA T	ATA I	ACA T	AGG R
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AGC S	AAG K	ATG M	AGA R	TTG L	TTA L	TTC F	GAT D	TTG L	AAT N	GCC A	GAA E
GTG V	AGG R	ACT T	GGT G	TAT Y	GAA E	ATT I	GGA G	AAG K	AAG K	ACG T	ACA T
TCC S	ACT T	TGG W	GAT D	TCA S	TCG S	GAG E	GCT A	GAA E	AAG K	TTA L	TTT F
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GAT D	TGG W	GAA E	CTT L	GGA G	AAT N	GAA E	CCG P	GAC D	CAC H	ACC T	TCA S
GCT A	CAC H	AAT N	TTA L	ACT T	GAA E	AAG K	CAG Q	GTT V	GGA G	GAA E	GAT D
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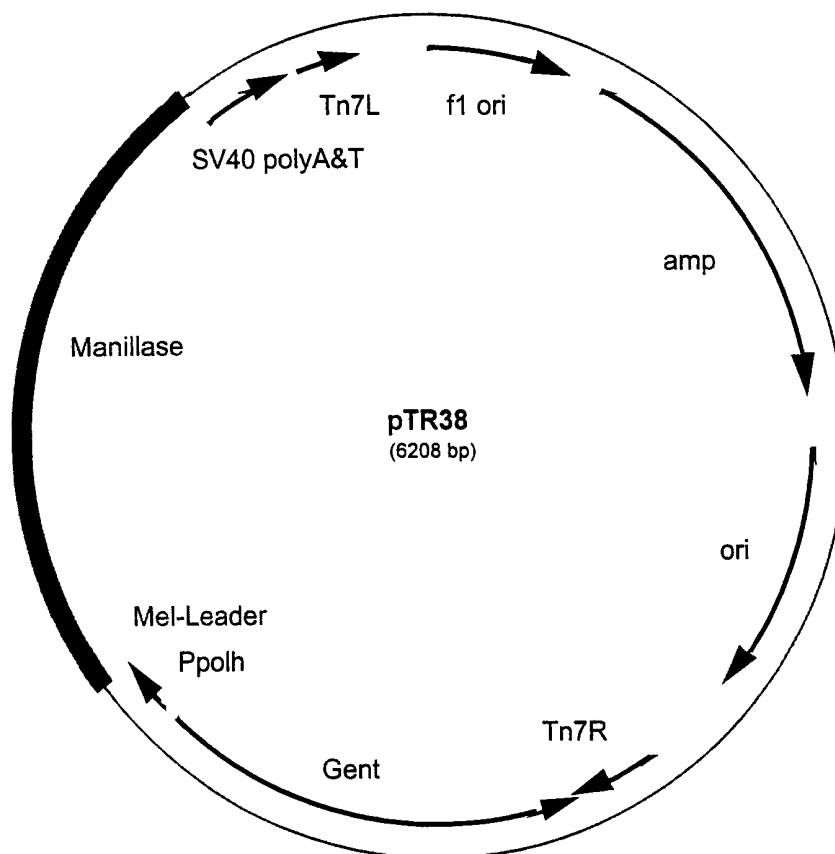
**Fig 10 (contnd)**

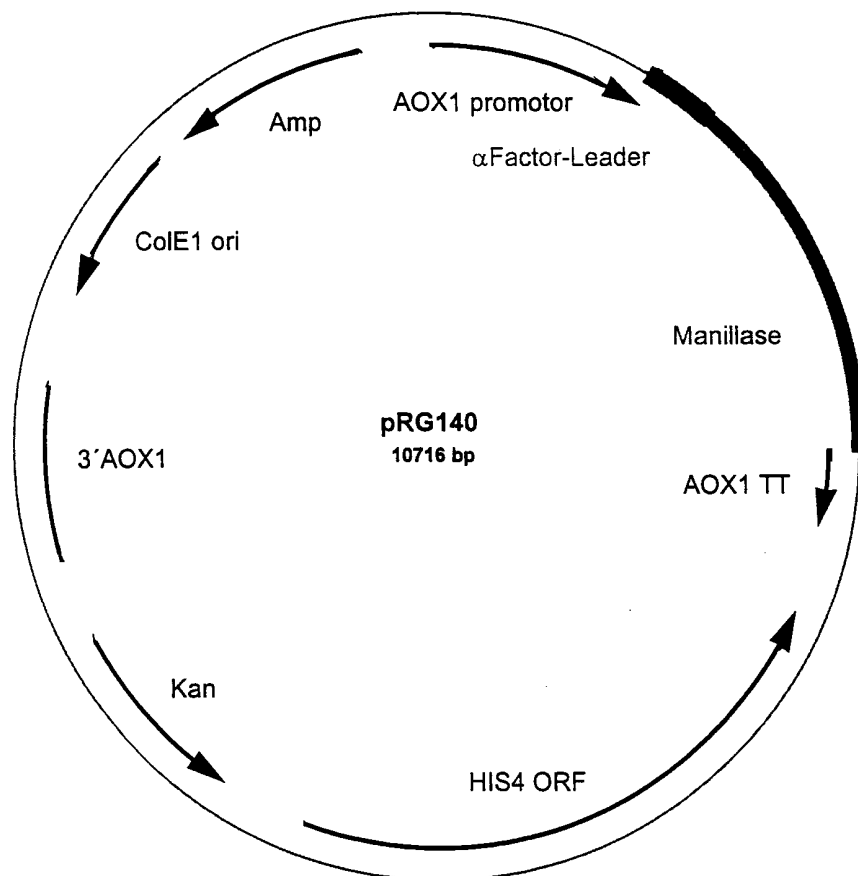
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GAC D	GGG G	GCA A	GGT G	GAC D	CTT L	GTA V	ACT T	GCT A	TTT F	ACA T	CTA L
CAC H	CAA Q	TAT Y	TAT Y	TTC F	GAT D	GGA G	AAC N	ACC T	TCT S	GAT D	GTA V
TCA S	ACA T	TAT Y	CTT L	GAT D	GCC A	TCA S	TAC Y	TTT F	AAA K	AAG K	CTG L
CAA Q	CAG Q	CTG L	TTT F	GAT D	AAA K	GTG V	AAA K	GAT D	GTT V	TTG L	AAA K
AAT N	TCT S	CCA P	CAT H	AAA K	GAC D	AAA K	CCA P	TTA L	TGG W	CTT L	GGA G
GAG E	ACA T	AGT S	TCT S	GGA G	TGC Y	AAC N	AGC S	GGC G	ACA T	AAA K	GAT D
GTA V	TCC S	GAT D	CGA R	TAT Y	GTT V	TCA S	GGA G	TTT F	CTA L	ACA T	TTA L
GAC D	AAG K	TTG L	GGT G	CTC L	AGT S	GCA A	GCC A	AAC N	AAT N	GTA V	AAG K
GTT V	GTT V	ATA I	AGA R	CAG Q	ACA T	ATA I	TAC Y	AAT N	GGA G	TAT Y	TAT Y
GGT G	CTC L	CTT L	GAT D	AAA K	AAC N	ACT T	TTA L	GAG E	CCA P	AAT N	CCT P
GAT D	TAC Y	TGG W	TTA L	ATG M	CAT H	GTT V	CAC H	AAT N	TCT S	TTG L	GTC V
GGA G	AAT N	ACA T	GTT V	TTT F	AAA K	GTT V	GAC D	GTT V	GGT G	GAT D	CCA P
ACT T	AAT N	AAA K	ACG T	AGA R	GTC V	TAT Y	GCA A	CAA Q	TGT C	ACC T	AAG K
ACA T	AAT N	AGC S	AAA K	CAC H	ACT T	CAA Q	GGC G	AAG K	TAT Y	TAC Y	AAG K
GGC G	TCT S	TTG L	ACA T	ATC I	TTT F	GCA A	CTT L	AAT N	GTT V	GGA G	GAT D

**Fig 10 (contnd)**

GAA E	GAA E	GTA V	ACG T	TTA L	AAG K	ATC I	GAT D	CAA Q	TAC Y	GGC G	GGT G
AAA K	AAA K	ATT I	TAT Y	TCA S	TAC Y	ATT I	CTG L	ACA T	CCT P	GAA E	GGA G
GGA G	CAA Q	CTT L	ACA T	TCA S	CAG Q	AAA K	GTT V	CTC L	TTG L	AAT N	GGA G
AAG K	GAA E	TTG L	AAC N	TTA L	GTG V	TCT S	GAT D	CAG Q	TTA L	CCA P	GAA E
CTA L	AAT N	GCA A	GAT D	GAA E	TCC S	AAA K	ACA T	TCT S	TTC F	ACC T	TTA L
TCC S	CCA P	AAG K	ACA T	TTT F	GGT G	TTT F	TTT F	GTT V	GTT V	TCC S	GAT D
GCT A	AAT N	GTT V	GAA E	GCA A	TGY C	AAR K	AAR K				

**Fig. 11:**

**Fig. 12:**

**Fig. 13:**

SEQUENCE LISTING

&lt;110&gt; Merck Patent GmbH

<120> Hyaluronidase from the Hirudinaria manillensis,  
isolation, purification and recombinant method of  
production

&lt;130&gt; Manillase

&lt;140&gt;

&lt;141&gt;

&lt;160&gt; 15

&lt;170&gt; PatentIn Ver. 2.1

&lt;210&gt; 1

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&lt;212&gt; PRT

&lt;213&gt; Leech

&lt;400&gt; 1

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1				5					10					15	

Ser	Glu	Ser	Phe	His	Gly	Val	Ala	Phe	Asp	Ala	Ser	Leu	Phe	Ser	Pro
			20					25					30		

Lys	Gly	Leu	Trp	Ser	Phe	Val	Asp	Ile	Thr	Ser	Pro	Lys	Leu	Phe	Lys
		35					40					45			

Leu	Leu	Glu	Gly	Leu	Ser	Pro	Gly	Tyr	Phe	Arg	Val	Gly	Gly	Thr	Phe
	50					55					60				

Ala	Asn	Trp	Leu	Phe	Phe	Asp	Leu	Asp	Glu	Asn	Asn	Lys	Trp	Lys	Asp
65					70					75					80

Tyr	Trp	Ala	Phe	Lys	Asp	Lys	Thr	Pro	Glu	Thr	Ala	Thr	Ile	Thr	Arg
				85					90					95	

Arg	Trp	Leu	Phe	Arg	Lys	Gln	Asn	Asn	Leu	Lys	Lys	Glu	Thr	Glu	Asp
		100					105						110		

Asp	Leu	Val	Lys	Leu	Thr	Lys	Gly	Ser	Lys	Met	Arg	Leu	Leu	Phe	Asp
	115						120					125			

Leu	Asn	Ala	Glu	Val	Arg	Thr	Gly	Tyr	Glu	Ile	Gly	Lys	Lys	Met	Thr
	130					135					140				

Ser	Thr	Trp	Asp	Ser	Ser	Glu	Ala	Glu	Lys	Leu	Phe	Lys	Tyr	Cys	Val
145				150						155					160

Ser	Lys	Gly	Tyr	Gly	Asp	Asn	Ile	Asp	Trp	Glu	Leu	Gly	Asn	Glu	Pro
				165					170					175	

Asp	His	Thr	Ser	Ala	His	Asn	Leu	Thr	Glu	Lys	Gln	Val	Gly	Glu	Asp
			180					185					190		

Phe	Lys	Ala	Leu	His	Lys	Val	Leu	Glu	Lys	Tyr	Pro	Thr	Leu	Asn	Lys
		195					200						205		

Gly Ser Leu Val Gly Pro Asp Val Gly Trp Met Gly Val Ser Tyr Val  
 210 215 220  
 Lys Gly Leu Ala Asp Gly Ala Gly Asp Leu Val Thr Ala Phe Thr Leu  
 225 230 235 240  
 His Gln Tyr Tyr Phe Asp Gly Asn Thr Ser Asp Val Ser Thr Tyr Leu  
 245 250 255  
 Asp Ala Thr Tyr Phe Lys Lys Leu Gln Gln Leu Phe Asp Lys Val Lys  
 260 265 270  
 Asp Val Leu Lys Asn Ser Gln His Lys Asp Lys Pro Leu Trp Leu Gly  
 275 280 285  
 Glu Thr Ser Ser Gly Tyr Asn Ser Gly Thr Lys Asp Val Ser Asp Arg  
 290 295 300  
 Tyr Val Ser Gly Phe Leu Thr Leu Asp Lys Leu Gly Leu Ser Ala Ala  
 305 310 315 320  
 Asn Asn Val Lys Val Val Ile Arg Gln Thr Ile Tyr Asn Gly Tyr Tyr  
 325 330 335  
 Gly Leu Leu Asp Lys Asn Thr Leu Glu Pro Asn Pro Asp Tyr Trp Leu  
 340 345 350  
 Met His Val His Asn Ser Leu Val Gly Asn Thr Val Phe Lys Val Asp  
 355 360 365  
 Val Ser Asp Pro Thr Asn Lys Ala Arg Val Tyr Ala Gln Cys Thr Lys  
 370 375 380  
 Thr Asn Ser Lys His Thr Gln Ser Arg Tyr Tyr Lys Gly Ser Leu Thr  
 385 390 395 400  
 Ile Phe Ala Leu Asn Val Gly Asp Glu Asp Val Thr Leu Lys Ile Asp  
 405 410 415  
 Gln Tyr Gly Gly Lys Lys Ile Tyr Ser Tyr Ile Leu Thr Pro Glu Gly  
 420 425 430  
 Gly Gln Leu Thr Ser Gln Lys Val Leu Leu Asn Gly Lys Glu Leu Lys  
 435 440 445  
 Leu Val Ser Asp Gln Leu Pro Glu Leu Asn Ala Asn Glu Ser Lys Thr  
 450 455 460  
 Ser Phe Thr Leu Ser Pro Lys Thr Phe Gly Phe Phe Val Val Ser Asp  
 465 470 475 480  
 Ala Asn Val Glu Ala Cys Lys Lys  
 485



<210> 2  
 <211> 1464  
 <212> DNA  
 <213> Leech

<220>  
 <221> CDS  
 <222> (1)..(1464)

<220>  
 <221> variation  
 <222> (667)..(669)  
 <223> Xaa = Tyr or Asn

<400> 2  
 aaa gag att gcc gtg aca att gac gat aag aat gtg att gca tct gcc 48  
 Lys Glu Ile Ala Val Thr Ile Asp Asp Lys Asn Val Ile Ala Ser Ala  
 1 5 10 15  
 agt ggg tct ttc ctt gga gtt gcc ttt gat gcg tct cta ttt tcg ccc 96  
 Ser Gly Ser Phe Leu Gly Val Ala Phe Asp Ala Ser Leu Phe Ser Pro  
 20 25 30  
 aag ggt ctt tgg agc ttt gtt gat att acc tct cca aaa ttg ttc aaa 144  
 Lys Gly Leu Trp Ser Phe Val Asp Ile Thr Ser Pro Lys Leu Phe Lys  
 35 40 45  
 ttg ctg gaa gga ctt tct cct gga tac ttc agg gtt ggc gga acg ttt 192  
 Leu Leu Glu Gly Leu Ser Pro Gly Tyr Phe Arg Val Gly Gly Thr Phe  
 50 55 60  
 gcc aat tgg ctg ttt ttt gac ttg gac gaa aat aat aag tgg aag gat 240  
 Ala Asn Trp Leu Phe Phe Asp Leu Asp Glu Asn Asn Lys Trp Lys Asp  
 65 70 75 80  
 tat tgg gct ttt aaa gac aaa acc ccc gaa act gcg aca ata aca agg 288  
 Tyr Trp Ala Phe Lys Asp Lys Thr Pro Glu Thr Ala Thr Ile Thr Arg  
 85 90 95  
 aga tgg ctg ttc aga aaa caa aat aat ctg aaa aag gag act ttt gac 336  
 Arg Trp Leu Phe Arg Lys Gln Asn Asn Leu Lys Lys Glu Thr Phe Asp  
 100 105 110  
 aat tta gtg aaa cta aca aag gga agc aag atg aga ttg tta ttc gat 384  
 Asn Leu Val Lys Leu Thr Lys Gly Ser Lys Met Arg Leu Leu Phe Asp  
 115 120 125  
 ttg aat gcc gaa gtg agg act ggt tat gaa att gga aag aag atg aca 432  
 Leu Asn Ala Glu Val Arg Thr Gly Tyr Glu Ile Gly Lys Lys Met Thr  
 130 135 140  
 tcc act tgg gat tca tcg gag gct gaa aag tta ttt aaa tat tgt gtg 480  
 Ser Thr Trp Asp Ser Ser Glu Ala Glu Lys Leu Phe Lys Tyr Cys Val  
 145 150 155 160  
 tca aaa ggt tac gga gac aat atc gat tgg gaa ctt gga aat gaa ccg 528  
 Ser Lys Gly Tyr Gly Asp Asn Ile Asp Trp Glu Leu Gly Asn Glu Pro  
 165 170 175  
 gac cac acc tca gct cac aat tta act gaa aag cag gtt gga gaa gat 576  
 Asp His Thr Ser Ala His Asn Leu Thr Glu Lys Gln Val Gly Glu Asp  
 180 185 190

ttt aaa gca ctg cat aaa gtt cta gag aaa tat cca act ctt aac aag	624
Phe Lys Ala Leu His Lys Val Leu Glu Lys Tyr Pro Thr Leu Asn Lys	
195 200 205	
gga tgc ctc gtt ggt cca gat gta ggg tgg atg ggc gtc agt wac gtc	672
Gly Ser Leu Val Gly Pro Asp Val Gly Trp Met Gly Val Ser Xaa Val	
210 215 220	
aag gga ttg gca gac gag gcr ggt gac cat gta ack gct ttt aca ctc	720
Lys Gly Leu Ala Asp Glu Xaa Gly Asp His Val Xaa Ala Phe Thr Leu	
225 230 235 240	
cac caa tat tat ttc gat gga aac acy tct gat gta tca ata tat ctt	768
His Gln Tyr Tyr Phe Asp Gly Asn Xaa Ser Asp Val Ser Ile Tyr Leu	
245 250 255	
gat gcc aca tac ttt aag aag ctg caa caa cta ttt gat aaa gtg aaa	816
Asp Ala Thr Tyr Phe Lys Lys Leu Gln Gln Leu Phe Asp Lys Val Lys	
260 265 270	
gat gtt ttg aaa gat tct cca cat aaa gac gaa cca tta tgg ctt gga	864
Asp Val Leu Lys Asp Ser Pro His Lys Asp Glu Pro Leu Trp Leu Gly	
275 280 285	
gaa aca agt tct gga tac aac agc ggc aca gaa gat gta tcc gat cga	912
Glu Thr Ser Ser Gly Tyr Asn Ser Gly Thr Glu Asp Val Ser Asp Arg	
290 295 300	
tat gtt tca gga ttt cta aca tta gac aag ttg ggt ctc agt gca gcc	960
Tyr Val Ser Gly Phe Leu Thr Leu Asp Lys Leu Gly Leu Ser Ala Ala	
305 310 315 320	
aac aat gta aag gtt gtt ata aga cag aca ata tac aat gga tat tat	1008
Asn Asn Val Lys Val Val Ile Arg Gln Thr Ile Tyr Asn Gly Tyr Tyr	
325 330 335	
ggt ctc ctt gac aaa aac act tta gag ccg aat ccg gat tac tgg tta	1056
Gly Leu Leu Asp Lys Asn Thr Leu Glu Pro Asn Pro Asp Tyr Trp Leu	
340 345 350	
atg cat gtt cat aat tct ttg gtc gga aat aca gtt ttt aaa gtt gac	1104
Met His Val His Asn Ser Leu Val Gly Asn Thr Val Phe Lys Val Asp	
355 360 365	
gtt agt gat cca act aat aaa gca aga gtt tac gcg caa tgt acc aaa	1152
Val Ser Asp Pro Thr Asn Lys Ala Arg Val Tyr Ala Gln Cys Thr Lys	
370 375 380	
aca aat agc aaa cat act caa agc aga tat tac aag ggc tct ttg aca	1200
Thr Asn Ser Lys His Thr Gln Ser Arg Tyr Tyr Lys Gly Ser Leu Thr	
385 390 395 400	
atc ttt gca ctt aat gtt gga gat gga gat gta acg tta aag atc ggt	1248
Ile Phe Ala Leu Asn Val Gly Asp Gly Asp Val Thr Leu Lys Ile Gly	
405 410 415	
caa tac agc ggt aaa aaa att tat tca tac att ctg aca cct gaa gga	1296
Gln Tyr Ser Gly Lys Lys Ile Tyr Ser Tyr Ile Leu Thr Pro Glu Gly	
420 425 430	
gga caa ctt aca tca cag aaa gtt ctc ttg aat gga aag gaa ttg aac	1344
Gly Gln Leu Thr Ser Gln Lys Val Leu Leu Asn Gly Lys Glu Leu Asn	
435 440 445	

tta gtg tct gat cag tta cca gaa cta aat gca gat gaa tcc aaa aca 1392  
 Leu Val Ser Asp Gln Leu Pro Glu Leu Asn Ala Asp Glu Ser Lys Thr  
           450                                  455                                  460

tct ttc acc tta tcc cca aag aca ttt ggt ttt ttt gtt gtt tcc gat 1440  
 Ser Phe Thr Leu Ser Pro Lys Thr Phe Gly Phe Phe Val Val Ser Asp  
 465                                  470                                  475                                  480

gct aat gtt gaa gca tgy aar aar 1464  
 Ala Asn Val Glu Ala Cys Lys Lys  
                                   485

<210> 3  
 <211> 488  
 <212> PRT  
 <213> Leech

<400> 3  
 Lys Glu Ile Ala Val Thr Ile Asp Asp Lys Asn Val Ile Ala Ser Ala  
   1                                  5                                  10                                  15

Ser Gly Ser Phe Leu Gly Val Ala Phe Asp Ala Ser Leu Phe Ser Pro  
                                   20                                  25                                  30

Lys Gly Leu Trp Ser Phe Val Asp Ile Thr Ser Pro Lys Leu Phe Lys  
                                   35                                  40                                  45

Leu Leu Glu Gly Leu Ser Pro Gly Tyr Phe Arg Val Gly Gly Thr Phe  
                                   50                                  55                                  60

Ala Asn Trp Leu Phe Phe Asp Leu Asp Glu Asn Asn Lys Trp Lys Asp  
   65                                  70                                  75                                  80

Tyr Trp Ala Phe Lys Asp Lys Thr Pro Glu Thr Ala Thr Ile Thr Arg  
                                   85                                  90                                  95

Arg Trp Leu Phe Arg Lys Gln Asn Asn Leu Lys Lys Glu Thr Phe Asp  
                                   100                                  105                                  110

Asn Leu Val Lys Leu Thr Lys Gly Ser Lys Met Arg Leu Leu Phe Asp  
                                   115                                  120                                  125

Leu Asn Ala Glu Val Arg Thr Gly Tyr Glu Ile Gly Lys Lys Met Thr  
                                   130                                  135                                  140

Ser Thr Trp Asp Ser Ser Glu Ala Glu Lys Leu Phe Lys Tyr Cys Val  
   145                                  150                                  155                                  160

Ser Lys Gly Tyr Gly Asp Asn Ile Asp Trp Glu Leu Gly Asn Glu Pro  
                                   165                                  170                                  175

Asp His Thr Ser Ala His Asn Leu Thr Glu Lys Gln Val Gly Glu Asp  
                                   180                                  185                                  190

Phe Lys Ala Leu His Lys Val Leu Glu Lys Tyr Pro Thr Leu Asn Lys  
                                   195                                  200                                  205

Gly Ser Leu Val Gly Pro Asp Val Gly Trp Met Gly Val Ser Xaa Val  
                                   210                                  215                                  220

Lys Gly Leu Ala Asp Glu Xaa Gly Asp His Val Xaa Ala Phe Thr Leu  
   225                                  230                                  235                                  240

His Gln Tyr Tyr Phe Asp Gly Asn Xaa Ser Asp Val Ser Ile Tyr Leu  
 245 250 255  
 Asp Ala Thr Tyr Phe Lys Lys Leu Gln Gln Leu Phe Asp Lys Val Lys  
 260 265 270  
 Asp Val Leu Lys Asp Ser Pro His Lys Asp Glu Pro Leu Trp Leu Gly  
 275 280 285  
 Glu Thr Ser Ser Gly Tyr Asn Ser Gly Thr Glu Asp Val Ser Asp Arg  
 290 295 300  
 Tyr Val Ser Gly Phe Leu Thr Leu Asp Lys Leu Gly Leu Ser Ala Ala  
 305 310 315 320  
 Asn Asn Val Lys Val Val Ile Arg Gln Thr Ile Tyr Asn Gly Tyr Tyr  
 325 330 335  
 Gly Leu Leu Asp Lys Asn Thr Leu Glu Pro Asn Pro Asp Tyr Trp Leu  
 340 345 350  
 Met His Val His Asn Ser Leu Val Gly Asn Thr Val Phe Lys Val Asp  
 355 360 365  
 Val Ser Asp Pro Thr Asn Lys Ala Arg Val Tyr Ala Gln Cys Thr Lys  
 370 375 380  
 Thr Asn Ser Lys His Thr Gln Ser Arg Tyr Tyr Lys Gly Ser Leu Thr  
 385 390 395 400  
 Ile Phe Ala Leu Asn Val Gly Asp Gly Asp Val Thr Leu Lys Ile Gly  
 405 410 415  
 Gln Tyr Ser Gly Lys Lys Ile Tyr Ser Tyr Ile Leu Thr Pro Glu Gly  
 420 425 430  
 Gly Gln Leu Thr Ser Gln Lys Val Leu Leu Asn Gly Lys Glu Leu Asn  
 435 440 445  
 Leu Val Ser Asp Gln Leu Pro Glu Leu Asn Ala Asp Glu Ser Lys Thr  
 450 455 460  
 Ser Phe Thr Leu Ser Pro Lys Thr Phe Gly Phe Phe Val Val Ser Asp  
 465 470 475 480  
 Ala Asn Val Glu Ala Cys Lys Lys  
 485

<210> 4  
 <211> 1464  
 <212> DNA  
 <213> Leech

<220>  
 <221> CDS  
 <222> (1)..(1464)

<220>  
 <221> variation  
 <222> (1348)..(1350)  
 <223> Xaa = Val or Met

&lt;400&gt; 4

aaa gag att gcc gtg aca att gac gat aag aat gtg att gca tct gcc	48
Lys Glu Ile Ala Val Thr Ile Asp Asp Lys Asn Val Ile Ala Ser Ala	
1 5 10 15	
agt gag tct ttc cat gga gtt gcc ttt gat gcg tct cta ttt tcg ccc	96
Ser Glu Ser Phe His Gly Val Ala Phe Asp Ala Ser Leu Phe Ser Pro	
20 25 30	
aag ggt ctt tgg agc ttt gtt gat att acc tct cca aaa ttg ttc aaa	144
Lys Gly Leu Trp Ser Phe Val Asp Ile Thr Ser Pro Lys Leu Phe Lys	
35 40 45	
ttg ctg gaa gga ctt tct cct gga tac ttc agg gtt ggc gga acg ttt	192
Leu Leu Glu Gly Leu Ser Pro Gly Tyr Phe Arg Val Gly Gly Thr Phe	
50 55 60	
gcc aat cgg ctg ttt ttt gac ttg gac gaa aat aat aag tgg aar gat	240
Ala Asn Arg Leu Phe Phe Asp Leu Asp Glu Asn Asn Lys Trp Lys Asp	
65 70 75 80	
tat tgg gct ttt aaa gac aaa acc ccc gaa act gcg aca ata aca agg	288
Tyr Trp Ala Phe Lys Asp Lys Thr Pro Glu Thr Ala Thr Ile Thr Arg	
85 90 95	
aga tgg ctg ttc aga aaa caa aat aat ctg aaa aag gag act ttt gac	336
Arg Trp Leu Phe Arg Lys Gln Asn Asn Leu Lys Lys Glu Thr Phe Asp	
100 105 110	
aat tta gtg aaa cta aca aag gga agc aag atg aga ttg tta ttc gat	384
Asn Leu Val Lys Leu Thr Lys Gly Ser Lys Met Arg Leu Leu Phe Asp	
115 120 125	
ttg aat gcc gaa gtg agg act ggt tat gaa att gga aag aag atg aca	432
Leu Asn Ala Glu Val Arg Thr Gly Tyr Glu Ile Gly Lys Lys Met Thr	
130 135 140	
tcc act tgg gat tca tcg gag gct gaa aag tta ttt aaa tat tgt gtg	480
Ser Thr Trp Asp Ser Ser Glu Ala Glu Lys Leu Phe Lys Tyr Cys Val	
145 150 155 160	
tca aaa ggt tac gga gac aat atc gat tgg gaa ctt ggg aat gga ccg	528
Ser Lys Gly Tyr Gly Asp Asn Ile Asp Trp Glu Leu Gly Asn Gly Pro	
165 170 175	
gac cac acc tca gct cac aat tta act gaa aag cag gtt gga gaa gat	576
Asp His Thr Ser Ala His Asn Leu Thr Glu Lys Gln Val Gly Glu Asp	
180 185 190	
ttt aaa gca ctg cat aaa gtt cta gag aaa tat cca act ctt aac aag	624
Phe Lys Ala Leu His Lys Val Leu Glu Lys Tyr Pro Thr Leu Asn Lys	
195 200 205	
gga tcg ctc gtt ggt cca gat gta ggg tgg atg ggc gtc agt tac gtc	672
Gly Ser Leu Val Gly Pro Asp Val Gly Trp Met Gly Val Ser Tyr Val	
210 215 220	
aag gga ttg gca gac gag gca ggt gac cat gta act gct ttt aca ctc	720
Lys Gly Leu Ala Asp Glu Ala Gly Asp His Val Thr Ala Phe Thr Leu	
225 230 235 240	

cac caa tat tat ttc gat gga aac acc tct gat gta tca ata tat ctt	768
His Gln Tyr Tyr Phe Asp Gly Asn Thr Ser Asp Val Ser Ile Tyr Leu	
245 250 255	
gat gcc aca tac ttt aag aag ctg caa caa cta ttt gat aaa gtg aaa	816
Asp Ala Thr Tyr Phe Lys Lys Leu Gln Gln Leu Phe Asp Lys Val Lys	
260 265 270	
gat gtt ttg aaa gat tct cca cat aaa gac aaa cca tta tgg ctt gga	864
Asp Val Leu Lys Asp Ser Pro His Lys Asp Lys Pro Leu Trp Leu Gly	
275 280 285	
gaa aca agt tct gga tac aac agc ggc aca gaa gat gta tcc gat cga	912
Glu Thr Ser Ser Gly Tyr Asn Ser Gly Thr Glu Asp Val Ser Asp Arg	
290 295 300	
tat gtt tca gga ttt cta aca tta gac aag ttg ggt ctc agt gca gcc	960
Tyr Val Ser Gly Phe Leu Thr Leu Asp Lys Leu Gly Leu Ser Ala Ala	
305 310 315 320	
aac aat gta aag gtt gtt ata aga cag aca ata tac agt gga tat tat	1008
Asn Asn Val Lys Val Val Ile Arg Gln Thr Ile Tyr Ser Gly Tyr Tyr	
325 330 335	
ggt ccc ctt gac aaa aac act tta gag cca aat ccg gat tac tgg tta	1056
Gly Pro Leu Asp Lys Asn Thr Leu Glu Pro Asn Pro Asp Tyr Trp Leu	
340 345 350	
atg cat gtt cat aat tct ttg gtc gga aat aca gtt ttt aaa gtt gac	1104
Met His Val His Asn Ser Leu Val Gly Asn Thr Val Phe Lys Val Asp	
355 360 365	
gtt agt gat cca act aat aaa gca aga gtt tac gcg caa tgt acc aaa	1152
Val Ser Asp Pro Thr Asn Lys Ala Arg Val Tyr Ala Gln Cys Thr Lys	
370 375 380	
aca aat agc aaa cat act caa agc aga tat tac aag ggc tct ttg aca	1200
Thr Asn Ser Lys His Thr Gln Ser Arg Tyr Tyr Lys Gly Ser Leu Thr	
385 390 395 400	
atc ttt gca ctt aat gtt gga gat gaa gat gta acg tta aag atc ggt	1248
Ile Phe Ala Leu Asn Val Gly Asp Glu Asp Val Thr Leu Lys Ile Gly	
405 410 415	
caa tac agc ggt aaa aaa att tat tca tac att ctg aca cct gaa gga	1296
Gln Tyr Ser Gly Lys Lys Ile Tyr Ser Tyr Ile Leu Thr Pro Glu Gly	
420 425 430	
gga caa ctt aca tca cag aaa gtt ctc ttg aat gga aag gaa ttg aac	1344
Gly Gln Leu Thr Ser Gln Lys Val Leu Leu Asn Gly Lys Glu Leu Asn	
435 440 445	
tta rtg tct gat cag tta cca caa cta aat gca gat gaa tcc aaa aca	1392
Leu Xaa Ser Asp Gln Leu Pro Gln Leu Asn Ala Asp Glu Ser Lys Thr	
450 455 460	
tct ttc acc tta tcc cca aag aca ttt ggt ttt ttt gtt gtt tcc gat	1440
Ser Phe Thr Leu Ser Pro Lys Thr Phe Gly Phe Phe Val Val Ser Asp	
465 470 475 480	
gct aat gtt gaa gca tgy aar aar	1464
Ala Asn Val Glu Ala Cys Lys Lys	
485	

<210> 5  
 <211> 488  
 <212> PRT  
 <213> Leech

<400> 5  
 Lys Glu Ile Ala Val Thr Ile Asp Asp Lys Asn Val Ile Ala Ser Ala  
     1                    5                    10                    15  
 Ser Glu Ser Phe His Gly Val Ala Phe Asp Ala Ser Leu Phe Ser Pro  
             20                    25                    30  
 Lys Gly Leu Trp Ser Phe Val Asp Ile Thr Ser Pro Lys Leu Phe Lys  
             35                    40                    45  
 Leu Leu Glu Gly Leu Ser Pro Gly Tyr Phe Arg Val Gly Gly Thr Phe  
     50                    55                    60  
 Ala Asn Arg Leu Phe Phe Asp Leu Asp Glu Asn Asn Lys Trp Lys Asp  
     65                    70                    75                    80  
 Tyr Trp Ala Phe Lys Asp Lys Thr Pro Glu Thr Ala Thr Ile Thr Arg  
             85                    90                    95  
 Arg Trp Leu Phe Arg Lys Gln Asn Asn Leu Lys Lys Glu Thr Phe Asp  
             100                    105                    110  
 Asn Leu Val Lys Leu Thr Lys Gly Ser Lys Met Arg Leu Leu Phe Asp  
     115                    120                    125  
 Leu Asn Ala Glu Val Arg Thr Gly Tyr Glu Ile Gly Lys Lys Met Thr  
     130                    135                    140  
 Ser Thr Trp Asp Ser Ser Glu Ala Glu Lys Leu Phe Lys Tyr Cys Val  
     145                    150                    155                    160  
 Ser Lys Gly Tyr Gly Asp Asn Ile Asp Trp Glu Leu Gly Asn Gly Pro  
             165                    170                    175  
 Asp His Thr Ser Ala His Asn Leu Thr Glu Lys Gln Val Gly Glu Asp  
             180                    185                    190  
 Phe Lys Ala Leu His Lys Val Leu Glu Lys Tyr Pro Thr Leu Asn Lys  
             195                    200                    205  
 Gly Ser Leu Val Gly Pro Asp Val Gly Trp Met Gly Val Ser Tyr Val  
     210                    215                    220  
 Lys Gly Leu Ala Asp Glu Ala Gly Asp His Val Thr Ala Phe Thr Leu  
     225                    230                    235                    240  
 His Gln Tyr Tyr Phe Asp Gly Asn Thr Ser Asp Val Ser Ile Tyr Leu  
             245                    250                    255  
 Asp Ala Thr Tyr Phe Lys Lys Leu Gln Gln Leu Phe Asp Lys Val Lys  
             260                    265                    270  
 Asp Val Leu Lys Asp Ser Pro His Lys Asp Lys Pro Leu Trp Leu Gly  
     275                    280                    285  
 Glu Thr Ser Ser Gly Tyr Asn Ser Gly Thr Glu Asp Val Ser Asp Arg  
     290                    295                    300

Tyr Val Ser Gly Phe Leu Thr Leu Asp Lys Leu Gly Leu Ser Ala Ala  
 305 310 315 320  
 Asn Asn Val Lys Val Val Ile Arg Gln Thr Ile Tyr Ser Gly Tyr Tyr  
 325 330 335  
 Gly Pro Leu Asp Lys Asn Thr Leu Glu Pro Asn Pro Asp Tyr Trp Leu  
 340 345 350  
 Met His Val His Asn Ser Leu Val Gly Asn Thr Val Phe Lys Val Asp  
 355 360 365  
 Val Ser Asp Pro Thr Asn Lys Ala Arg Val Tyr Ala Gln Cys Thr Lys  
 370 375 380  
 Thr Asn Ser Lys His Thr Gln Ser Arg Tyr Tyr Lys Gly Ser Leu Thr  
 385 390 395 400  
 Ile Phe Ala Leu Asn Val Gly Asp Glu Asp Val Thr Leu Lys Ile Gly  
 405 410 415  
 Gln Tyr Ser Gly Lys Lys Ile Tyr Ser Tyr Ile Leu Thr Pro Glu Gly  
 420 425 430  
 Gly Gln Leu Thr Ser Gln Lys Val Leu Leu Asn Gly Lys Glu Leu Asn  
 435 440 445  
 Leu Xaa Ser Asp Gln Leu Pro Gln Leu Asn Ala Asp Glu Ser Lys Thr  
 450 455 460  
 Ser Phe Thr Leu Ser Pro Lys Thr Phe Gly Phe Phe Val Val Ser Asp  
 465 470 475 480  
 Ala Asn Val Glu Ala Cys Lys Lys  
 485

<210> 6  
 <211> 1464  
 <212> DNA  
 <213> Leech

<220>  
 <221> CDS  
 <222> (1)..(1464)

<400> 6  
 aaa gag att gcc gtg aca att gac gat aag aat gtg att gca tct gtc 48  
 Lys Glu Ile Ala Val Thr Ile Asp Asp Lys Asn Val Ile Ala Ser Val  
 1 5 10 15  
 agt gag tct ttc cat gga gtt gcc ttt gat gcg tct cta ttc tcg ccc 96  
 Ser Glu Ser Phe His Gly Val Ala Phe Asp Ala Ser Leu Phe Ser Pro  
 20 25 30  
 aag ggt cct tgg agc ttt gtt aat att acc tct cca aaa ttg ttc aaa 144  
 Lys Gly Pro Trp Ser Phe Val Asn Ile Thr Ser Pro Lys Leu Phe Lys  
 35 40 45  
 ttg ctg gaa gga ctt tct cct gga tac ttc agg gtt ggc gga acg ttt 192  
 Leu Leu Glu Gly Leu Ser Pro Gly Tyr Phe Arg Val Gly Gly Thr Phe  
 50 55 60



gcc aat tgg ctg ttt ttt gac ttg gac gaa aat aat aag tgg aag gat	240
Ala Asn Trp Leu Phe Phe Asp Leu Asp Glu Asn Asn Lys Trp Lys Asp	
65 70 75 80	
tat tgg gct ttt aaa gac aaa acc ccc gaa act gcg aca ata aca agg	288
Tyr Trp Ala Phe Lys Asp Lys Thr Pro Glu Thr Ala Thr Ile Thr Arg	
85 90 95	
aga tgg ctg ttc aga aaa caa aat aat ctg aaa aag gag act ttt gac	336
Arg Trp Leu Phe Arg Lys Gln Asn Asn Leu Lys Lys Glu Thr Phe Asp	
100 105 110	
gat tta gtg aaa cta aca aag gga agc aag atg aga ttg tta ttc gat	384
Asp Leu Val Lys Leu Thr Lys Gly Ser Lys Met Arg Leu Leu Phe Asp	
115 120 125	
ttg aat gcc gaa gtg agg act ggt tat gaa att gga aag aag acg aca	432
Leu Asn Ala Glu Val Arg Thr Gly Tyr Glu Ile Gly Lys Lys Thr Thr	
130 135 140	
tcc act tgg gat tca tcg gag gct gaa aag tta ttt aaa tat tgt gtg	480
Ser Thr Trp Asp Ser Ser Glu Ala Glu Lys Leu Phe Lys Tyr Cys Val	
145 150 155 160	
tca aaa ggt tac gga gac aat atc gat tgg gaa ctt gga aat gaa ccg	528
Ser Lys Gly Tyr Gly Asp Asn Ile Asp Trp Glu Leu Gly Asn Glu Pro	
165 170 175	
gac cac acc tca gct cac aat tta act gaa aag cag gtt gga gaa gat	576
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Phe Lys Ala Leu His Lys Val Leu Glu Lys Tyr Pro Thr Leu Asn Lys	
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Lys Gly Leu Ala Asp Gly Ala Gly Asp Leu Val Thr Ala Phe Thr Leu	
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His Gln Tyr Tyr Phe Asp Gly Asn Thr Ser Asp Val Ser Thr Tyr Leu	
245 250 255	
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Asp Ala Ser Tyr Phe Lys Lys Leu Gln Gln Leu Phe Asp Lys Val Lys	
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Asp Val Leu Lys Asn Ser Pro His Lys Asp Lys Pro Leu Trp Leu Gly	
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Ile Phe Ala Leu Asn Val Gly Asp Glu Glu Val Thr Leu Lys Ile Asp  
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Gln Tyr Gly Gly Lys Lys Ile Tyr Ser Tyr Ile Leu Thr Pro Glu Gly  
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Gly Gln Leu Thr Ser Gln Lys Val Leu Leu Asn Gly Lys Glu Leu Asn  
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Leu Val Ser Asp Gln Leu Pro Glu Leu Asn Ala Asp Glu Ser Lys Thr  
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Ser Phe Thr Leu Ser Pro Lys Thr Phe Gly Phe Phe Val Val Ser Asp  
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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 00/05181

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/52 C12N9/26 A61K38/43

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)

IPC 7 C12N A61K

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 practical, search terms used)

BIOSIS, EMBASE, CHEM ABS Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 193 330 A (BIOPHARM LTD) 3 September 1986 (1986-09-03) cited in the application the whole document ---	1-4,6,8, 9,16,17, 20
A	JONES C. P. AND SAWYER R. T.: "Heparin inhibits mammalian, but not leech hyaluronidase." THROMBIN RESEARCH, vol. 55, no. 6, 1989, pages 791-796, XP000953413 the whole document ---	1-20
A	US 5 827 721 A (FORMBY BENT ET AL) 27 October 1998 (1998-10-27) column 6, line 24 - line 61; example 1 -----	7



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

### \* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search

1 November 2000

Date of mailing of the international search report

15/11/2000

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# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 00/05181

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0193330 A	03-09-1986	DE 3688710 A DE 3688710 T US 4820516 A	26-08-1993 18-11-1993 11-04-1989
US 5827721 A	27-10-1998	US 5747027 A WO 9631596 A	05-05-1998 10-10-1996