



The present invention relates to novel inhibitors of human tryptase, to their isolation from leeches, to nucleotide sequences encoding the novel inhibitor molecules or fragments thereof, to vectors containing the coding sequence thereof, to host cells transformed with such vectors, to the recombinant production of the inhibitors, to pharmaceutical compositions containing the novel inhibitor molecules, and to their use in diagnosis and therapy.

Tryptase is a tetrameric member of the family of trypsin-like serine proteinases. Tryptase is expressed virtually exclusively by mast cells [Castells Irani, 1987] and stored in large amounts in their secretory granules, constituting ~23% of the total cellular protein [Schwartz Lewis Austen, 1981]. Following activation of mast cells, tryptase is rapidly released into the extracellular space together with other preformed mediators (e.g. histamine, chymase, and proteoglycans) [Schwartz Lewis Seldin, 1981; Caughey Lazarus, 1988]. Elevated levels have been found

- in the plasma of patients with mastocytosis, after systemic anaphylaxis [Schwartz Metcalfe, 1987; Schwartz Yunginger, 1989], and during the systemic response after aspirin challenge of patients with aspirin-sensitive asthma [Bosso Schwartz, 1991],
- in bronchoalveolar lavage fluid of patients with asthma [Broide Gleich, 1991; Bousquet Chanez, 1991; Wenzel Fowler, 1988], interstitial lung diseases [Walls Bennett, 1991], and after antigen challenge of allergic patients [Castells, 1988; Butrus, 1990],
- in the skin blister fluid after cutaneous antigen challenge in patients with atopic and allergic skin disease [Shalit Schwartz, 1990; Atkins Schwartz, 1990],
- in nasal lavage fluid after local antigen challenge of patients with seasonal allergic rhinitis [Juliussen Holmberg, 1991],
- in the crevicular fluid of patients with gingivitis



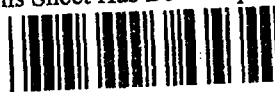
and periodontitis [Cox Eley, 1989, J Period Res; Eley Cox, 1992, J Dent], and

- in the lesional skin of patients with psoriasis [Harvima Naukkarinen, 1989].

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In vitro studies have provided considerable evidence that tryptase is directly involved in the pathogenesis of mast cell related disorders. For example, tryptase has been suggested as a pathogenetic mediator of asthma as it increases the
10 contractility of airway smooth muscle [Sekizawa, 1989] and inactivates vasoactive intestinal peptide, thereby destroying its potent bronchodilatory action [Tam Caughey, 1990; Tam Franconi, 1990; Franconi, 1989]. In addition, tryptase has been shown to be a potent mitogen for fibroblasts, suggesting
15 its involvement in the pulmonary fibrosis in asthma and interstitial lung diseases [Ruoss Hartmann, 1991; Hartmann Ruoss, 1992]. Tryptase has also been implicated in the pathogenesis of arthritis and periodontal disease, as it activates prostromelysin (= MMP-3) which in turn activates
20 collagenase, thereby initiating the destruction of cartilage and periodontal connective tissue, respectively [Gruber Marchese, 1989; Gruber Schwartz, 1990; Cox Eley, 1989, J Period Res; Eley Cox, 1992, J Dent]. Tryptase may also promote blood clotting disorders by inactivating the procoagulant function of
25 high molecular weight kininogen [Maier Spragg, 1983] and by cleaving fibrinogen [Schwartz Bradford Littman, 1985].

Human tryptase is virtually unique among the serine proteinases as it is fully catalytically active in plasma and in the
30 extracellular space [Schwartz Bradford, 1986; Goldstein Leong, 1992]. Tryptase is not inhibited by the naturally occurring antiproteinases regulating the activity of other trypsin-like serine proteinases such as mucus-proteinase inhibitor (=antileukoprotease or HUSI-I), antithrombin III, alpha₁-
35 proteinase inhibitor, alpha₂-macroglobulin, or C₁-esterase inhibitor [Alter Kramps, 1990; Smith Hougland, 1984; Schwartz Bradford, 1986; Harvima Schechter, 1988; Cromlish Seidah, 1987]. Furthermore, although tryptase has been known for over



10 years, inhibitors derived from non-human species or produced by peptide synthesis or recombinant technologies have not yet been described. Thus, tryptase is not affected by hirudin [Alter Kramps, 1990], aprotinin, ovomucoid inhibitor, soybean
5 and lima bean trypsin inhibitor [Butterfield Weiler, 1990; Cromlish Seidah, 1987; Harvima Schechter, 1988], ecotin [Chung Ives, 1983], and the recombinant Kunitz-domain of the Alzheimer beta-amyloid precursor-protein [Sinha Dovey, 1990].

10 Although tryptase is inhibited by the general inhibitors of trypsin-like proteinases such as diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride and tosyl-L-lysine chloromethyl ketone [Smith Hougland, 1984; Harvima Schechter, 1988], these
15 compounds are unsuitable for *in vivo* and even for most *in vitro* applications due to their high toxicity and/or low stability. Furthermore, the only other inhibitors known to affect tryptase, the peptide-arginine aldehydes leupeptin and antipain [Cromlish Seidah, 1987], and certain benzamidin derivatives
20 [Stürzebecher Prasa, 1992; Caughey, 1993] are of limited usefulness as they are relatively unspecific, and/or inhibit tryptase only with moderate affinities (K_i values for the complexes in the micromolar range).

The problem of the present invention is therefore to provide a
25 potent and efficient inhibitor of the human proteinase tryptase.

As illustrated in further detail below the present problem can be solved by providing an inhibitor polypeptide obtainable from
30 medical leech *Hirudo medicinalis*.

Brief description of the figures:

35 Fig.1: Isolation of the leech-derived tryptase inhibitor by cation-exchange chromatography using SP-Sephadex. Dialysed leech extract was applied and the column was washed until the absorption of the effluent returned to baseline. Desorption was achieved with 20 mM NaP,

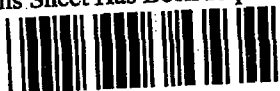
500 mM NaCl (pH 8.0). Fractions containing inhibitory active material (marked with a bar) were pooled.

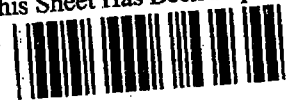
5 **Fig.2:** Affinity-chromatography of the leech-derived tryptase inhibitor using anhydrotrypsin-Sepharose. The pooled eluate from the SP-Sephadex chromatography was applied, the column washed extensively and eluted with 100 mM KCl/HCl (pH 2.1). Fractions containing inhibitory active material (marked with a bar) were collected and neutralised immediately by addition of 1 M Tris.

10
15 **Fig.3:** Cation-exchange chromatography of the leech-derived tryptase inhibitor using a Mono S FPLC column. After dialysis against 20 mM NaP (pH 8.0) the pooled eluate from the anhydrotrypsin affinity-chromatography was applied, the column washed, and eluted using a linear gradient from 60 to 240 mM NaCl. The fractions containing inhibitory active material (marked with a bar) were pooled.

20
25 **Fig.4:** SDS-PAGE of the isolated leech-derived tryptase inhibitor under reducing conditions. Lane 1 = dialysed leech extract; lane 2 = eluate from the SP-Sephadex column; lane 3 = eluate from the anhydrotrypsin affinity-chromatography; lane 4 = eluate from Mono S cation exchange chromatography. The molecular weight markers (lane 5) are from top to bottom: ovalbumin (43 kD), carbonic anhydrase (29 kD), β -lactoglobulin (18.4 kD), lysozyme (14.3 kD), bovine trypsin inhibitor (6200 D), and insulin β -chain (3400 D).

30
35 **Fig.5:** Reversed phase HPLC of the isolated leech-derived tryptase inhibitor. The elution time and the absorption of the effluent at 206 nm are given on the abscissa and the ordinate, respectively. The two





peaks demonstrate the presence of at least two forms.

Fig.6: Tryptic fragmentation of the two species of the leech-derived tryptase inhibitor separated by reversed phase HPLC (see Fig. 5). Lower tracing: HPLC-tracing of the tryptic digest of the peak eluting at 25 min in Fig. 5; Upper tracing: HPLC-tracing of the tryptic digest of the peak eluting at 29 min. The elution profiles differ only in the peaks representing the C-terminal peptides (marked by arrows).

Fig.7: Mass spectroscopy of the two species of the leech-derived tryptase inhibitor separated by HPLC (see Fig. 5). a) The mass spectrum of the HPLC peak eluting at 25 min demonstrates the presence of 2 forms with a mass of 4340 (form A; left peaks) and 4396 (form B; right peaks), respectively. b) The mass spectrum of the HPLC peak eluting at 29 min shows a third form (form C) with a mass of 4738.

Fig.8: Sequence determination of the leech-derived tryptase-inhibitor. The bars represent the overlapping fragments used in deducing the amino acid sequence. The solid bars denote the sequence obtained from the HPLC peak eluting at 25 min (see Fig. 5), and the hatched bar the additional sequence obtained from the HPLC peak eluting at 29 min. N-terminal = sequence obtained from the native inhibitor; Red/T = sequence obtained after reduction and tryptic fragmentation; Ox/T/ChT = sequence obtained after oxidation and tryptic/chymotryptic fragmentation.

Fig.9: Inhibition of human tryptase by the leech-derived tryptase inhibitor. Tryptase (0.59 nM) was preincubated with increasing concentrations of the leech-derived tryptase inhibitor (0-40 nM) at 37°C for 25 min, and the reaction was initiated by the

addition of substrate tos-Gly-Pro-Arg-pNa. The resulting steady state velocities were measured over 3.5 min. The values given are the quotient of the velocity in the presence of the inhibitor divided by the velocity in the absence of the inhibitor.

Fig.10: Effect of the leech-derived tryptase inhibitor on the tryptase-induced cleavage of vasoactive intestinal peptide (VIP). VIP was incubated with tryptase in the presence of increasing concentrations of the leech derived tryptase inhibitor. Thereafter, the amount of VIP cleaved was quantified by reversed phase HPLC. The values given are the quotient of the velocity in the presence of the inhibitor divided by the velocity in the absence of the inhibitor.

Fig.11: Design, DNA and amino acid sequence of synthetic rLDTI form-C gene. (a) Design of the synthetic rLDTI form-C master gene. The introduced restriction sites are shown. (b) Nucleotide and corresponding amino acid sequence of the rLDTI form-C master gene. Brackets and numbers indicate the synthetic oligonucleotides used to assemble the gene. (c) Modification of rLDTI form-C master gene by cassette mutagenesis

Fig.12: (a) Plasmid map of pRM 5.1.5. (b) Expression vector pRM 9.1.4. A synthetic gene for rLDTI-form C was ligated into the purified yeast secretion vector pVT102U/ α , cleaved with XbaI and HindIII. Arrows indicate the direction of transcription; ADH-p, the ADH1 gene promoter; mat, the α -mating factor leader gene; ADH-t, the 3' region of the ADH1 gene including a transcription terminator signal; Ura-3, the Ura gene; amp-R, the ampicillin-resistance gene; the E.coli ori, yeast ori (2 μ -ori) and the intergenic region of phage f1 (f1-ori).





Fig.13: SDS/PAGE analysis of fermentation supernatant and purified rLDTI form-C. lane 1 = low-molecular mass-markers; lane 2 = fermentation supernatant of yeast strain H005 after 96 hours of cultivation (80 μ l); lane 3 = purified rLDTI-form C (2 μ g).

Fig.14: HPLC analysis of purified rLDTI form-C. Reverse phase HPLC on a RP 18 column was performed with 7,4 nmol (35 μ g) purified inhibitor. A linear gradient of 0-60% (by vol.) acetonitrile formed from 0,1% (by vol.) trifluoroacetic acid in acetonitrile and 0,1 % (by vol.) trifluoroacetic acid was used. The flow rate was adjusted to 1.0 ml/min and the absorbance in the effluent was monitored at 206 nm.

Fig.15: Plasmid map of expression vector pRM 3.1.10

Fig.16: Plasmid map of expression vector pRM 4.1.4

Fig.17: Plasmid map of expression vector pRM 11.1.4.

According to a first embodiment the present invention relates to purified inhibitor molecules of human tryptase. The novel inhibitors are polypeptides obtainable from extracts of leeches, as for example the medical leech *Hirudo medicinalis*. The invention also relates to the functional equivalents of the inhibitor molecules showing tryptase inhibitor activity, and to the pharmaceutically acceptable salts of the inhibitors.

The inhibitor molecules of the present invention are characterized by their ability to inhibit human tryptase with a K_i value in the range of approximately 0.1 to 10 nM, leaving the proteases involved in the human blood coagulation cascade substantially unaffected.

Preferably tryptase inhibitors are provided which are characterized by the following amino acid sequence (position 1: N-terminal amino acid Lys):

Lys-Lys-Val-Cys-Ala-Cys-Pro-Lys-Ile-Leu 10
 Lys-Pro-Val-Cys-Gly-Ser-Asp-Gly-Arg-Thr 20
 Tyr-Ala-Asn-Ser-Cys-Ile-Ala-Arg-Cys-Asn 30
 Gly-Val-Ser-Ile-Lys-Ser-Glu-Gly-Ser-Cys 40
 Pro-Thr-X 42

wherein X = H (SEQ ID No:1), Gly (SEQ ID NO:2) or Gly-Ile-Leu-Asn (SEQ ID NO:3).

The present invention also encompasses genetic variants, alleles or functional equivalents of the above-mentioned sequence, of which one or more of the amino acids are substituted (conservative or non-conservative) or deleted, or to which one or more amino acids are added without substantially affecting the tryptase inhibitor activity.

Conservative substitutions encompass for example substitutions within the following groups of amino acids (one letter code): G,A; V,I,L; D,E; N,Q; K,R; and S,Y,N. Preferably the amino acid addition or deletion is performed at the N- and/or C-terminal end of the above-mentioned sequence. The functional equivalents with altered and/or improved specificity and/or inhibitory efficiency can easily be prepared by a person of ordinary skill applying usual methods of peptide synthesis, or applying methods well known in the field of molecular biology, as for example site directed mutagenesis or undirected mutagenesis (e.g. using a phage display system).

Functional equivalents of the inhibitor of the present invention are for example those comprising the amino acid sequence

R^1 -Cys-Pro-Lys-Ile-Leu
 Lys-Pro-Val-Z-Gly-Ser-Asp-Gly-Arg-Thr
 Tyr-Ala-Asn-Ser-Cys-Ile-Ala- R^2

wherein

the N-terminal residue R^1 represents Ala- or Cys-Ala-;
 the C-terminal residue R^2 represents -Arg or -Arg-Cys; and
 Z defines any, preferably any naturally occurring, amino acid.





Moreover, based on the teaching of the present invention a person of ordinary skill will be enabled to prepare fragments of the natural forms of the inhibitor still showing the desired tryptase inhibiting activity.

5 The naturally occurring forms of the claimed inhibitor molecules may either be isolated from leech, preferably the medical leech *Hirudo medicinalis*, or may be prepared by peptide synthesis or recombinant DNA technology.

10 According to a further aspect of the present invention a synthetic gene coding for form C (SEQ ID NO:3) of the leech derived tryptase inhibitor (LDTI-C) was designed, cloned and expressed in *Escherichia coli* and *Saccharomyces cerevisiae*. The
15 coding fragment was assembled via 6 oligonucleotides, it contains linker sequences, stop codons and selected recognition sites for further modifications, for example by cassette mutagenesis. Strong expression of the recombinant form C inhibitor (rLDTI-C) was found using *Saccharomyces cerevisiae*
20 secretion vector pVT102U/alpha and strain S-78. The secreted material was isolated by centrifugation and cross-flow filtration, and further purified by cation exchange chromatography, it is inhibitorily active and about 85% pure. Amino acid sequencing showed that rLDTI-C is predominantly
25 correct processed at the junction between the alpha mating factor leader peptide and the first amino acids of LDTI-C; only minor amounts of truncated forms were detected. The far UV-CD spectrum of the recombinant molecule is typical for a folded protein containing secondary structural elements. The molecular
30 mass of HPLC purified material is 4738 ± 4 Da as determined by electrospray ionization mass spectrometry. The rLDTI-C displays equilibrium dissociation constants with bovine trypsin and human tryptase which are nearly identical to those of the
35 natural one. The expected expression products encoded within the expression vector were also identified in vitro, using a S30 transcription translation system.

The proteins presented in this invention are the first



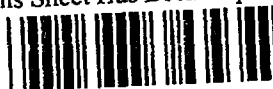
compounds known to be efficient inhibitors of tryptase. Thus, the leech-derived tryptase inhibitors reduce the catalytic activity of tryptase, the K_i value of the enzyme-inhibitor complex being in the nanomolar range. Moreover, the inhibitors affect not only the tryptase-induced cleavage of the peptide-nitroanilid substrate used as a tool to determine the activity of the proteinase *in vitro*. They also affect the cleavage of vasoactive intestinal peptide (VIP) and of kininogen, representatives of the peptides and proteins thought to be biologically relevant substrates of tryptase. In addition, the inhibitors efficiently diminish the tryptase-induced growth of human keratinocytes - an example of the direct cellular effects of tryptase - without causing apparent cytotoxic or other side effects.

Besides having a high affinity for tryptase, the leech-derived tryptase inhibitors are highly specific. Thus, with the exception of the pancreatic proteinases trypsin and chymotrypsin, other serine proteinases are not or only marginally inhibited, the K_i values for the enzyme-inhibitor complexes being at least 200 times higher than for the complex with tryptase. Their specificity is illustrated by the lacking effect on the blood coagulation *ex vivo*, verifying that the proteinases involved in the coagulation cascade are not affected.

Thus, the leech-derived tryptase inhibitors of the present invention will allow for the first time the inhibition of tryptase with high affinity and specificity. Consequently, the inhibitors provide the prospect to effectively block pathophysiologic events involving the cleavage of proteins and peptides and/or the activation of cells by tryptase.

Therefore, it is an object of the current invention to apply the inhibitors as probes in the diagnosis well as drugs in the therapy of tryptase- and mast cell related diseases.

According to a further preferred embodiment of the present



invention nucleotide sequences, as for example DNA and RNA sequences, are provided which encode a polypeptide with tryptase inhibitor activity or fragments thereof. Preferably polynucleotide molecules comprising the following general nucleotide sequence (SEQ ID NO:4) are provided:

5'

1 AARAARGTNTGYGCNTGYCCNAARATHYTNAARCCNGTNTGYGGNWSNGA

51 YGGNMGNACNTAYGCNAAYSNTGYATHGCMGNTGYAAAYGGNGTNWSNA

101 THAARWSNGARGGNWSNTGYCCNACNX

3'

wherein R denotes A or G; M denotes A or C; W denotes A or T; S denotes C or G; Y denotes C or T; H denotes A, C, or T; N denotes any nucleotide; and X denotes -OH (SEQ ID NO:4), GGN (SEQ ID NO:5) or GGN ATH YTN AAY (SEQ ID NO:6). The invention also relates to the complementary strand thereof; and the DNA sequences which hybridize, preferably under stringent conditions, to the afore-mentioned DNA sequence.

Preferably the polynucleotides of the present invention comprise a nucleotide sequence substantially corresponding to nucleotide residues 1 to 149, or more preferably 7 to 144 of SEQ ID NO:7; or fragments thereof comprising at least 15 to 21 consecutive nucleotides of SEQ ID NO:7. Within the scope of the invention are also complementary polynucleotides comprising a nucleotide sequence substantially corresponding to nucleotide residues 1 to 149, or preferably 10 to 147 of SEQ ID NO:8; or fragments thereof comprising at least 15 to 21 consecutive nucleotides of SEQ ID NO:8. Preferably, these fragments are tryptase inhibitor specific or functional derivatives of these nucleotide sequences.

According to a further embodiment the present invention refers to an oligonucleotide which hybridizes, preferably under stringent conditions, to a nucleotide sequence encoding a polypeptide with tryptase inhibitor activity. Preferably, this oligonucleotide comprises a nucleotide sequence which is substantially complementary to the nucleotide sequence from residue 22 to residue 87 of SEQ ID NO:7.



Another embodiment of the invention refers to polynucleotides encoding a polypeptide with tryptase inhibitor activity which polynucleotides being obtainable by hybridizing, preferably under stringent conditions, with an oligonucleotide as specified above; as well as to polypeptides encoded by said polynucleotides. Suitable stringent conditions can be determined easily by one skilled in the art.

Also within the scope of the present invention are the polynucleotide sequence of SEQ ID NO:9, and functional equivalents thereof.

The present invention also encompasses vector molecules for the transformation of eucaryotic or procaryotic hosts, comprising a DNA molecule as defined above. For example, the vector may be a virus or a plasmid containing the inhibitor encoding DNA sequence in functional relation with suitable transcriptional and translational regulatory sequences well known in the art. The coding sequence may also be linked to suitable autonomously replicating sequences (ARS). Suitable host cells may be transformed with a vector containing the tryptase inhibitor coding sequence, and the inhibitor produced by the host cells may be expressed and isolated in a suitable way from the cell culture.

A further embodiment of the invention is a polypeptide expression cassette comprising a promoter operably linked to a DNA sequence coding for the polypeptide and to a DNA sequence containing transcription termination signals. In hosts capable of secreting expressed polypeptides, the expression cassette preferably comprises a promoter operably linked to a first DNA sequence encoding a signal peptide linked in the proper reading frame to a second DNA sequence coding for the inventive polypeptide, and a DNA sequence containing transcription termination signals.

In a preferred embodiment, the promoter, the signal sequence and the terminator are recognized by the yeast expression



system.

promoters suitable for expression in a certain host are well known. Examples are the promoter of the TRP1 gene, the ADHI or ADHII gene, acid phosphatase (PH05) gene, CUP1 gene, iso-
5 cytochrome c gene, or a promoter of the genes coding for glycolytic enzymes, such as TDH3, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a shortened version of GAPDH (GAPFL), 3-phosphoglycerate kinase (PGK), hexokinase, pyruvate
10 decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, invertase and glucokinase genes, or a promoter of the yeast mating pheromone genes coding for the a- or α -factor, can be used. Preferred
15 vectors of the present invention contain, e.g., promoters with transcriptional control that can be turned on or off by variation of the growth conditions, e.g. the promoter of the PH05 or the CUP1 gene. For example, the PH05 promoter can be repressed or derepressed at will, solely by increasing or
20 decreasing the concentration of inorganic phosphate in the medium and the CUP1 promoter can be turned on by the addition of Cu^{2+} -ions to the medium, e.g., in the form of a copper salt. Especially preferred are the GAPDH and the yeast CUP1 promoter.

25 The DNA sequence encoding a signal peptide ("signal sequence"), e.g. a yeast signal peptide, is preferably derived from a gene, e.g. a yeast gene, coding for a polypeptide which is ordinarily secreted. Yeast signal sequences are, for example, the signal and prepro sequences of the yeast invertase (SUC2), α -factor,
30 pheromone peptidase (KEX1), "killer toxin" and repressible acid phosphatase (PH05) genes and the glucoamylase signal sequence from *Aspergillus awamori*. Additional sequences, such as pro- or spacer-sequences which may carry specific processing signals can also be included in the constructions to facilitate
35 accurate processing of precursor molecules. For example, the processing signals contain a Lys-Arg residue, which is recognized by a yeast endopeptidase located in the Golgi membranes. The preferred signal sequences according to the

present invention are those of the yeast PH05 gene, the α -factor and of the yeast invertase gene (SUC2).

5 A DNA sequence containing transcription termination signals, e.g. yeast transcription termination signals, is preferably the 3' flanking sequence of a gene, e.g. a yeast gene, which contains proper signals for transcription termination and polyadenylation. The preferred flanking sequence is that of the yeast PH05 and the α -factor gene.

10 The DNA coding for the polypeptide according to the invention may be isolated from a gene bank of the natural host (the medical leech *Hirudo medicinalis*) by methods known in the art or synthesized by PCR using, e.g., the preferred codon usage of
15 the host.

The promoter, the DNA sequence coding for the signal peptide, the DNA sequence coding for the polypeptide and the DNA sequence containing transcription termination signals are
20 operably linked to each other, i.e. they are juxtaposed in such a manner that their normal functions are maintained. The array is such that the promoter effects proper expression of the signal sequence-polypeptide gene complex, the transcription termination signals effect proper termination of transcription
5 and polyadenylation. The signal sequence is linked in the proper reading frame to the polypeptide gene in such a manner that the last codon of the signal sequence is directly linked to the first codon of the gene for the polypeptide. The yeast promoter is preferably joined to the signal sequence between
10 the major mRNA start and the ATG naturally linked to the promoter gene. The signal sequence has its own ATG for translation initiation. The junction of these sequences may, for example, be effected by means of synthetic oligo-deoxynucleotide linkers carrying the recognition sequence of an endonuclease. Examples for related expression cassettes are described e.g. in EP-A-341215.

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Preferred expression cassettes comprise the CUP1 or the GAPDH promoter, the α -factor or the yeast invertase leader sequence, the tryptase inhibitor gene and the α -factor terminator.

- 5 Especially preferred expression cassette comprise a recombinant DNA molecule as described in Example 9 or a functional fragment or derivative thereof.

10 A further embodiment of the invention concerns a recombinant plasmid comprising a polypeptide expression cassette as described above.

Apart from the polypeptide expression cassette the expression plasmids according to the invention can comprise a DNA segment
15 originating from two-micron DNA containing the origin of replication or, if a two-micron DNA free strain of yeast is used, total two-micron DNA. The latter type of plasmids is preferred. For example, the plasmids according to the invention may contain the complete two-micron DNA in an uninterrupted
20 form, i.e. two-micron DNA is cleaved once with a restriction endonuclease, the linearized DNA is linked with the other components of the vector prior to recircularization. The restriction site is chosen such that normal function of the REP1, REP2 and FLP genes and of the ORI, STB, IR1 and IR2 sites
25 of two-micron DNA as well as small "FLP recognition target" (FRT) sites, located near the center of each inverted repeat (IR) at which the FLP recombinase acts, is maintained. Optionally, the restriction site is chosen such that the D gene of two-micron DNA is kept intact, too. Suitable restriction
30 sites are, for example, the unique PstI site located within the D gene and the unique HpaI and SnaBI sites located outside of all of said genes and sites. However, it is likewise possible to insert the expression cassette and further components (cf. below) at different (such as two) restriction sites, especially
35 those mentioned above, within two-micron DNA.

Preferably, the expression plasmids according to the invention include one or more, especially one or two, selective genetic

markers, e.g. a marker for yeast and a marker and (except for symmetric two-micron like hybrid vectors) an origin of replication for a bacterial host, especially *Escherichia coli*.

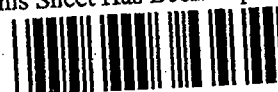
- 5 As to the selective gene markers, any marker gene can be used which facilitates the selection for transformants due to the phenotypic expression of the marker gene. Suitable markers are, for example, those expressing antibiotic resistance or, in the case of auxotrophic yeast mutants, genes which complement host
- 10 lesions. Corresponding genes confer, for example, resistance to the antibiotics G418, hygromycin or bleomycin or provide for prototrophy in an auxotrophic yeast mutant, for example the URA3, LEU2, LYS2, HIS3 or TRP1 gene.
- 15 As the amplification of the expression plasmids is conveniently done in a prokaryote, such as *E. coli*, a prokaryote, e.g. *E. coli*, genetic marker and a prokaryote, e.g. *E. coli*, replication origin are included advantageously. These can be obtained from corresponding prokaryotic plasmids, for example
- 20 *E. coli* plasmids, such as pBR322 or a pUC plasmid, for example pUC18 or pUC19, which contain both prokaryotic, e.g. *E. coli*, replication origin and genetic marker conferring resistance to antibiotics, such as ampicillin.
- 25 A suitable vector for transforming yeast cells is plasmid pRM 9.1.4 as deposited with the DSM and having the accession number DSM 9271.

Further preferred vector molecules are

- 30 pRM11.1.4 as deposited with the DSM and having the accession number DSM 9272;
- pRM5.1.5 as deposited with the DSM and having the accession number DSM 9270;
- pRM4.1.4 as deposited with the DSM and having the accession
- 35 number DSM 9269; and
- pRM3.1.10 as deposited with the DSM and having the accession number DSM 9268.

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The vector may also be selected from pHE175, pHE175R, pHE177 and pHE177R as disclosed in the experimental part below.

According to a further embodiment of the present invention a method of preparing a tryptase inhibitor is provided, comprising the steps of

- a) obtaining an extract of a leech, preferably of the medicinal leech *Hirudo medicinalis*, and
- b) purifying the extract by dialysis and column chromatography.

Preferably the crude extract is dialyzed against a buffer of low ionic strength. Subsequently the dialyzed extract is purified by cation exchange chromatographie, bio-specific chromatography, as for example anhydrotrypsin-sepharose affinity chromatography, and a further cation exchange chromatography step. Further experimental details are illustrated in the experimental part below. Modifications of the claimed process can easily be designed by a person of ordinary skill which are still encompassed by the scope of the present invention.

The invention also refers to methods of preparing recombinant tryptase inhibitor and to recombinant tryptase inhibitors obtainable by these methods. According to one preferred embodiment this method comprises

- a) transforming a prokaryotic or eukaryotic host with a vector as defined above;
- b) inducing the expression of the tryptase inhibitor encoding sequence;
- c) recovering the expression product; and optionally
- d) removing from the obtained product peptide fragments not required for tryptase inhibitor activity and/or optionally renaturing the product.

The coding sequence may also be expressed applying a suitable transcription translation system, as for example the S30 transcription translation system.

According to another embodiment of the present invention prokaryotic and eukaryotic hosts transformed with a vector encoding a tryptase inhibitor, and variants and mutants thereof are provided.

Suitable hosts are of prokaryotic or eukaryotic origin. Examples are bacterial, fungal, plant or insect cells. Preferred hosts are bacterial and fungal cells such as *E. coli* or fungi like *Saccharomyces cerevisiae*, *Aspergillus niger*, *Aspergillus nidulans* or *Neurospora crassa*.

Preferred yeast strains are those mentioned above, e.g. strains of *S. cerevisiae* which have been cured of the endogenous two-micron plasmid ("cir⁰ strains") and especially strains which are singly or multiply deficient in yeast proteases; and/or, in the case the CUP1 promoter is used, yeast strains containing 1-3 additional copies of the chromosomal CUP1 gene.

A wide variety of proteinases, like those mentioned, have been characterized in the yeast *Saccharomyces cerevisiae* [Achstetter et al. (1985)]. Mutants lacking activity of most of these proteases have been isolated and studied biochemically. The consequences of the absence of certain proteases were elucidated and some properties proved to be useful for the production of heterogeneous proteins. The proteases which are lacking in the yeast strains according to the invention do not perform indispensable functions in the cell metabolism; therefore mutations which completely destroy the activity of these proteins are not lethal. For example, the yeast strain lack one or more proteases selected from the group of carboxypeptidases ysc⁻, yscB, yscA, yscY and yscS. Methods for the production of such yeast strains are described, for example, in EP-A-40170 and EP-A-341215.

The transformation of host with the hybrid plasmids according to the invention may be accomplished according to methods known in the art.

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A preferred embodiment refers to a eukaryotic host derived from *S. cerevisiae* S-78 as deposited with the DSM and having the accession number DSM 9273 and to variants and mutants thereof capable of producing a tryptase inhibiting molecule.

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According to another preferred embodiment of the present invention pharmaceutical compositions are provided, comprising a tryptase inhibiting amount of a polypeptide as defined above, prepared from leech extracts or obtained for example by
10 expression of a recombinant tryptase inhibitor encoding gene, optionally in combination with a pharmaceutically acceptable carrier or diluent.

These compositions can be used in particular in the case of the
15 indications mentioned herein, if they are administered, e.g. parenterally (such as intravenously, intracutaneously, intramuscularly or subcutaneously), orally, by inhalation or topically. The dosage depends essentially on the specific method of administration and on the purpose of the treatment or
20 prophylaxis. The size of the individual doses and the administration programme can best be determined based on an individual assessment of the relevant case. The methods required to determine the relevant factors are familiar to the expert. Normally, in the case of injection, the therapeutically
25 active quantity of the compounds according to the invention is in the dosage range of approximately 0.005 to approximately 1 mg/kg of body weight. The range from approximately 0.01 to approximately 0.05 mg/kg of body weight is preferred.

30 Administration is by intravenous, intramuscular or subcutaneous injection. Consequently, depending on the method of application, pharmaceutical preparations for parenteral administration contain approximately 0.5 to approximately 10 mg of the compound according to the invention per individual dose.
35 In addition to the active ingredient, these pharmaceutical compositions usually also contain a buffer, e.g. a phosphate buffer, intended to keep the pH value between approximately 3.5 and 7, and, furthermore, sodium chloride, mannitol or sorbitol

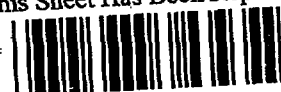
in order to adjust isotonicity. They can be in freeze-dried or dissolved form, wherein the solutions can advantageously contain an antibacterial preserving agent, e.g. 0.2 to 0.3 % of 4-hydroxybenzoic acid methyl ester or ethyl ester.

5

A preparation for topical application can be in the form of an aqueous solution, lotion or jelly, an oily solution or suspension or a fatty or, particularly, emulsion ointment. A preparation in the form of an aqueous solution is obtained, e.g. by dissolving the substance according to the invention or a therapeutically useful salt thereof in an aqueous buffer solution of pH 4 to 6.5 and, if desired, adding one or more further substance thereto. The concentration of the active ingredient is approximately 0.08 to approximately 1.5 mg, preferably 0.25 to 1.0 mg, in approximately 10 ml of a solution or 10 g of a jelly.

An oily form of application for topical administration is obtained, e.g. by suspending the substance according to the invention or a therapeutically useful salt thereof in an oil, optionally with the addition of swelling agents, such as aluminium stearate, and/or surface-active agents (surfactants) the HLB value (hydrophilic-lipophilic balance) of which is less than 10, such as fatty acid monoesters of polyhydric alcohols, e.g. glycerol monostearate, sorbitan monolaurate, sorbitan monostearate or sorbitan monooleate. A greasy ointment is obtained, e.g. by suspending the substance according to the invention or the salts in a spreadable greasy base, optionally with the addition of a surfactant having an HLB value of less than 10. An emulsion ointment is obtained by trituration of an aqueous solution of the substance according to the invention or the salts in a soft spreadable greasy base with the addition of a surfactant, the HLB value of which is less than 10. All of these forms of topical application can also contain a preserving agent. The concentration of the active ingredient is approximately 0.08 to approximately 1.5 mg, preferably 0.25 to 1.9 mg in approximately 10 g of the matrix.

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This invention also relates to the bioanalytical use of the compounds according to the invention and the salts thereof for the analytical determination of trypase and the preparations serving to this end, containing the substances according to the invention, e.g. solid mixtures and above all solutions, in particular aqueous solutions. In addition to a specific quantity or concentration of the substances according to the invention (also in the form of a salt), these can also contain inert adjuvants, e.g. those mentioned above with reference to the injection preparations, which have, e.g. a stabilising and/or preserving function.

According to a further embodiment the present invention is concerned with the use of a tryptase inhibitor as defined above in diagnosing functional tryptase and mast cell related disorders. Especially preferred is the use for preparing pharmaceutical compositions for the treatment of asthma, intestinal lung disease, arthritis, periodontal disease, allergic disorders, blood clotting disorders, skin disorders and psoriasis.

EXPERIMENTAL PART

1. MATERIALS

a) Leech Extracts: Extracts from the medical leech *Hirudo medicinalis* were a gift from Plantorgan, Germany. The leech extracts may also be prepared on the basis of the disclosure of EP-A-0 207 956 and the references cited therein.

b) Enzymes and Substrates: Proteases were obtained as follows: bovine trypsin, porcine pancreatic kallikrein, and porcine pancreatic elastase (Sigma; Deisenhofen, Germany); human factor Xa (Boehringer Mannheim; Mannheim, Germany); human neutrophil elastase, human thrombin, human urokinase, and bovine chymotrypsin (Medor; Herrsching, Germany); human plasmin, and human plasma kallikrein (Kabi; Essen, Germany);

human cathepsin G (Calbiochem; Bad Soden, Germany).

5 Tryptase was purified from human lung tissue to apparent homogeneity using a modification of described methods [Smith Hougland, 1984; Schwartz Lewis Austen, 1981; Harvima Schechter, 1988].

10 The following substrates were purchased: Bz-Ile-Glu-Gly-Arg-pNA (Novabiochem; Bad Soden, Germany); Suc-Ala-Ala-Ala-pNA (Bachem; Heidelberg, Germany); D-Pro-Phe-Arg-pNA, and D-Val-Leu-Arg-pNA (Kabi; Essen, Germany); Suc-Val-Pro-Phe-pNA, and Pyr-Gly-Arg-pNA (Medor; Herrsching, Germany); MeO-Suc-Ala-Ala-Pro-Val-pNA (Sigma; Munich, Germany). Tos-Gly-Pro-Arg-pNA was obtained from Boehringer Mannheim, Medor, and Sigma.

15 (Tos = tosyl; Suc = succinyl; pNA = p-nitroanilide).

Vasoactive intestinal peptide (VIP) was purchased from Calbiochem (Bad Soden, Germany), and bovine lung heparin from Sigma. Bdelein B was a gift from E. Fink (Klinische Chemie und

20 Klinische Biochemie, Chirurgische Klinik, LMU; Munich, Germany).

c) Column materials: SP-Sephadex ®, cyanogen bromide-activated Sepharose ® 4B and Mono S ® HR 5/5 were obtained from

25 Pharmacia (Freiburg, Germany).

Anhydrotrypsin was prepared from trypsin, affinity-purified by a modification of the methods described by Ako [Ako Foster Ryan, 1972], and immobilised onto cyanogen bromide-activated

30 Sepharose 4B according to the guidelines of Pharmacia.

d) Cell culture: Media, foetal calf serum, and antibiotics were obtained from Biochrom (Berlin, Germany). The human keratinocyte cell line HaCaT [Boukamp Petrussevska, 1988] was

35 obtained from N. Fusenig, German Cancer Research Center (DKFZ; Heidelberg, Germany). [Methyl-³H]thymidine was purchased from Amersham Buchler (Braunschweig, Germany).

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2. METHODS

2.1. Purification of the leech-derived tryptase inhibitor

5 a) Chromatography on SP-Sephadex ®: Leech extract (~3.5 g) was dissolved in deionised water (77 ml) and dialysed against 20 mM NaP (pH 8.0) over night at 4°C. The dialysed material was applied onto a SP-Sephadex ® column (1.6 x 20 cm) equilibrated with the same buffer. The column was washed at a flow rate of 1 ml/min until the optical density (280 nm) of the effluent
10 reached baseline, and eluted with 20 mM NaP, 500 mM NaCl (pH 8.0). Fractions containing inhibitory active material were collected and pooled.

15 b) Affinity-chromatography on anhydrotrypsin-Sepharose ®: The pooled material from the cation exchange chromatography (~20 ml) was applied onto an anhydrotrypsin-Sepharose column (1.6 x 3.6 cm) equilibrated with 20 mM NaP (pH 8.0). Approximately 90% of the inhibitory active material applied was bound; the remainder in the flow-through was collected for
20 rechromatography. After extensive washing of the column (~10 column volumes) elution was started by addition of 100 mM KCl/HCl (pH 2.1) at a flow rate of 0.3 ml/min. Fractions were collected and neutralised immediately by addition of 1 M Tris. The pooled eluate was dialysed against 20 mM NaP (pH 8.0) over
25 night at 4°C.

c) Chromatography on Mono S®: The dialysed eluate from the affinity chromatography was bound on a Mono S cation exchange column (0.5 x 5 cm) equilibrated with 20 mM NaP (pH 8.0). The
30 column was washed with the same buffer (~20 ml), and eluted using a gradient from 60 to 240 mM NaCl in 50 column volumes at flow rate of 1 ml/min. Fractions containing inhibitory active material were pooled (~5 ml), aliquoted, and stored at -20°C.

2.2. Standard Analytical Methods

35 a) Protein Assay: Protein concentrations were determined using the bicinchoninic acid procedure [Smith Krohn, 1985] with bovine serum albumin as standard.

b) **Electrophoresis:** Electrophoretic analysis of reduced and denatured protein was performed using 10-20% SDS-polyacrylamide gradient gels as described by Laemmli [Laemmli, 1970].

Proteins were detected after silver staining [Heukeshoven, 1985].

c) **HPLC:** Samples (~1 nmol) were loaded onto a Lichrospher RP 8 reversed phase column (120 x 4 mm; Merck) and eluted using a linear gradient from 0 % to 30 % acetonitrile in 0.1 % TFA at a flow rate of 1 ml/min.

d) **Sequence analysis:**

Reduction and S- β -pyridylethylation: S- β -pyridylethylation was carried out essentially as described by Friedman et al.

[Friedman Krull, 1970]. The inhibitor (1-2 nmol) was dissolved in 100 μ l buffer (6 M guanidinium-HCl, 0.25 M Tris-HCl, 1 mM EDTA, 5 % (v/v) β -mercaptoethanol; pH 8.5) and incubated overnight at room temperature. After addition of 5 μ l 4-vinylpyridine and incubation for 90 min, the reaction was stopped by acidification with formic acid. The S-pyridinethylated inhibitor was desalted by reversed phase chromatography on an Aquapore RP 300 column (2.1 x 30 mm; Applied Biosystems, Pfungstadt, Germany).

Oxidation of the inhibitor: A mixture of formic acid (45 μ l) and hydrogen peroxide (30 %; 5 μ l) was preincubated for 1 h at RT. Thereafter, the inhibitor (1-2 nmol) was dissolved in this mixture. After incubation for 1 h at 4°C, the reaction was stopped by dilution with 1 ml deionised water and lyophilisation.

Fractionation: The inhibitor (1 nmol) was incubated with trypsin and/or chymotrypsin (both sequencing grade; Boehringer Mannheim) in 100 μ l of 1 M ammoniumhydrogencarbonate buffer (pH 8.0) for 14 h at 37°C. An enzyme/inhibitor ratio of 1:40 was used. The reaction was terminated by acidification with formic acid, and fragments were separated by HPLC.



Amino acid sequence analysis: Automated amino-acid sequencing was performed using a gas-phase sequencer 473A (Applied Biosystems, Weiterstadt, Germany).

5 e) *Sequence comparison:* The MIPSX-database (Martinsrieder Institut für Proteinsequenzen am Max-Planck-Institut für Biochemie, Martinsried, Germany) was searched using the Lipman & Pearson fast protein searching algorithm FASTP [Lipman Pearson, 1985]. Alignments were optimised using CLUSTAL
10 [Higgins Sharp, 1988].

f) *Amino acid analysis:* Samples of oxidised inhibitor were hydrolysed under vacuum in 5.7 M hydrochloric acid at 110°C for 20 h and analysed on a Biotronik LC 5000 high performance
15 analyser system (Puchheim, Germany).

g) *Determination of the molecular mass:* The molecular mass of the HPLC-purified inhibitor (50 µM) was determined using a tandem quadrupole instrument API III (Sciex, Thornhill,
20 Ontario, Canada). The instrument was calibrated with the ammonium adduct ions of polypropylene glycol.

h) *Inhibitory activity:* During the purification procedure the inhibitor was followed by measurements of its effect on the
25 amidolytic activity of tryptase. Therefore, samples were incubated with tryptase (0.59 nM) in 50 mM Tris/HCl (pH 7.6), 150 mM NaCl, 50 µg/ml bovine lung heparin, and 0.1 % (w/v) bovine serum albumin for 25 min at 37°C. The assay was started by addition of the substrate tos-Gly-Pro-Arg-pNa at a final
30 concentration of 0.1 mM. The released nitroaniline was monitored spectrometrically at 405 nm for 3.5 min using a UVIKON 930 photometer (Kontron; Eching, Germany).

One inhibition unit (IU) was defined as the amount of inhibitor
35 which reduces the substrate hydrolysis by 30%.

i) *Titration of the inhibitor:* The concentration of inhibitory active leech-derived tryptase inhibitor was determined by





titration with trypsin. Therefore, bovine pancreatic trypsin was standardised by active-site titration using p-Nitrophenyl p'-guanidinobenzoate [Chase Shaw, 1970]. The concentration of active inhibitor was calculated assuming a 1:1 interaction
5 between the inhibitor and trypsin.

k) Determination of equilibrium constants: To determine the specificity of the inhibitor, its effect on the amidolytic activity of various serine proteinases (see Tab. 5) was
10 determined. Therefore, proteinases were incubated with the inhibitor (0.2 μ M) for 15 and 30 min under the conditions indicated in Tab. 5. The residual enzyme activity was measured after addition of a suitable substrate.

15 Equilibrium dissociation constants (K_i) for the complexes of the inhibitor with individual proteases were determined essentially as described by Bieth [Bieth, 1980]. Briefly, increasing concentrations of the inhibitor were incubated with a constant concentration of the enzyme; the time necessary to reach
20 equilibration of the enzyme-inhibitor complex was determined for each protease in preliminary experiments. Substrate was then added, and the residual enzyme activity measured. K_i -values were calculated by fitting the steady state velocities to the equation for tight binding inhibitors [Morrison, 1969]
25 using non-linear regression analysis.

l) Coagulation assay: The prothrombin time according to Quick and the partial thromboplastin time were measured using an Amelung KC 10 coagulometer (Lemgo, Germany) and the reagent
30 sets from Behringwerke AG (Marburg, Germany) according to the guidelines of the manufacturers.

m) Cleavage of vasoactive intestinal peptide (VIP): Trypsase (4.8 nM) was preincubated with different concentrations of the
35 leech-derived trypsin inhibitor in 100 mM Tris (pH 7.4), 140 mM NaCl, 50 μ g/ml heparin at 37°C for 25 min. Vasoactive intestinal peptide (VIP; 24 μ M, final concentration) was then added. After incubation for additional 1 to 10 min, the



reaction was stopped by acidification with acetic acid. The remaining VIP and the fragments generated were quantified using HPLC.

- 5 n) Growth of human keratinocytes: For growth studies the human keratinocyte cell line HaCaT, a spontaneously transformed cell line maintaining characteristics of differentiated keratinocytes [Boukamp Petrussevska, 1988], was utilised. HaCaT cells were plated in 24-well tissue culture plates
- 10 (Falcon; Becton Dickinson, Heidelberg, Germany) at a density of 10^4 cells/cm² in a medium containing 90% Dulbecco's modified Eagles' medium, 10% foetal calf serum, and 50 µg/ml gentamicin. Cells were incubated at 37°C in 5% CO₂. After 24 h, the cells were washed twice with serum-free Dulbecco's modified Eagles'
- 15 medium, and fresh serum-free medium containing 7.8 µg/ml heparin alone or medium containing the agonists and/or the inhibitor was added. After 48 h, the cells were washed two times again, and fresh serum-free medium containing 1 µCi/ml ³H-thymidin was added. After additional 2 h, the cells were
- 20 washed three times with ice-cold Dulbecco's PBS, and incorporated ³H-thymidin was precipitated by 10% trichloroacetic acid. After solubilisation of the precipitate in 0.1 N NaOH, 1% SDS, the incorporated radioactivity was determined by liquid scintillation counting (beta counter model LS 1800, Beckman
- 25 Instruments, Munich, Germany). For growth studies other keratinocyte cell lines may be applied as well.

Example 1: Isolation of the leech-derived trypsin-inhibitor

30

- 3.5 g of lyophilised leech extract was dissolved in water, dialysed against 20 mM NaP (pH 8.0), and applied onto a SP-Sephadex ® cation exchange column (see Method Section). The bulk of the protein (~98%) and of the trypsin-inhibitory
- 35 activity was found in the flow-through, whereas the leech-derived trypsin inhibitor was bound to the column. After elution of the column with 500 mM NaCl (Fig. 1), the inhibitor was separated from non-trypsin inhibiting proteins by

subsequent affinity-chromatography on anhydrotrypsin-sepharose (Fig. 2). Final purification was achieved by Mono S $\oplus$ cation-exchange chromatography (Fig. 3). The data of the isolation procedure are summarised in Table 1.

Table 1: Purification of the leech-derived tryptase inhibitor.

Purification step	Volume [ml]	Protein [mg]	Activity [IU]	Specific activity [IU/mg]	Yield [%]	Purification (fold)
Dialysis	91.5	2311	124000	50	-	-
SP-Sephadex	19.7	35.8	103000	2890	83	58
Anhydro-trypsin-Sephadex	15.2	2.11	47100	22300	38	446
Dialysis	14.5	1.75	36300	20700	29	414
Mono S	4.5	0.08	9910	122000	8	2440

3.5 g of lyophilised leech extract was used as starting material. One inhibitory unit (IU) was defined as the amount of inhibitor reducing the amidolytic activity of tryptase by 30% (see Methods).

The isolated leech-derived tryptase inhibitor was homogeneous according to SDS-PAGE and N-terminal sequence analysis (Fig. 4 and 8). However, two species were separated by reversed phase HPLC (Fig. 5). Subsequent amino acid sequencing after tryptic fragmentation, amino acid analysis, and mass spectroscopy (Tab. 2, Fig. 6 and 7) demonstrated that the two species comprise three forms differing only in their C-terminal sequence. Thus, forms B (43 aa) and C (46 aa) differ from the shortest form A (42 aa) by a C-terminal extension of -GLY and -GLY-ILE-LEU-ASN,



respectively. The results obtained for the 3 forms are compared in Tab. 3.

5

Table 2: Amino acid analysis of the two species of the leech-derived tryptase inhibitor separated by HPLC (see Fig. 5).

10

15

20

25

Amino acid	Form A/B ¹⁾	Form C
Asx	3.49 (3)	4.18 (4)
Thr	2.17 (2)	2.22 (2)
Ser	4.76 (5)	4.86 (5)
Glx	1.43 (1)	1.26 (1)
Gly	5.3 (4/5) ²⁾	5.62 (5)
Ala	3.43 (3)	3.18 (3)
Cys	5.68 (6)	5.24 (6)
Val	2.82 (3)	2.94 (3)
Ile	3.15 (3)	4.19 (4)
Leu	1.08 (1)	2.03 (2)
Tyr	n.d. (1)	n.d. (1)
Lys	5.03 (5)	5.00 (5)
Arg	1.7 (2)	1.74 (2)
Pro	3.01 (3)	3.81 (3)

The values given in brackets are the values calculated from the amino acid sequence. ¹⁾ Forms A and B have not been separated;

²⁾ The sequence of the forms A and B contain 4 and 5 glycins, respectively; n.d. not determined

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Table 3: Summary of the characterisation of the three forms of the leech-derived tryptase-inhibitor.

	Form A	Form B	Form C
C-terminal Sequence	CPT	CPTG	CPTGILN
Molecular mass determined by mass spectroscopy	4340	4396	4738
Molecular mass calculated from the sequence ¹⁾	4341	4398	4738
Elution time on reversed phase HPLC (see Fig. 5)	25 min ²⁾		29 min
Elution time of the C-terminal peptide of the tryptic digest (see Fig. 6)	13 min ²⁾		23 min
Inhibitory activity	+ ²⁾		+

1) assuming three disulphide bonds

2) Forms A and B have not been separated

The N-terminal 35 amino acid residues of the leech derived inhibitor were determined by sequencing the native inhibitor. The primary structure was completed and verified using overlapping peptides generated after modification and tryptic and/or chymotryptic fragmentation (Fig. 8).

Sequence comparisons demonstrate that the leech-derived tryptase inhibitor is a non-classical Kazal-type serine proteinase inhibitor. The highest degree of similarity was found to Bdellin B [Fink Rehm, 1986]; in the sequence section common to both inhibitors (amino acids 1-40), 19 of 40 (47.5 %) amino acids are identical (Tab. 4). Despite the high sequence identity to the leech-derived tryptase inhibitor, Bdellin B, an inhibitor also isolated from the medical leech, does not affect

tryptase (unpublished observations).

Table 4: Comparison of the amino acid sequences of the leech-derived tryptase inhibitor and Bdellin B-3 [Fink Rehm, 1986].

		1	5	10	5	20	5	30	5	40
10	Tryptase-Inhibitor	KKVCACPKILKPVCSDGRTYANSCIARCNGVSIKSEGSC								
	Bdellin B-3	DTECVCTKELHRVCGSDGVTYDNECLATCHGASVAHDHAC								
		.. *.*.* *..***** **.*.*.* *.*.*. . .*								
15	Tryptase-Inhibitor	-----PT								
	Bdellin B-3	EGHEEHVDEHGEDHD								
		..								

(* = identical residues; . = homologues amino acids)

Example 2: Specificity of the leech-derived inhibitor

25 The leech-derived tryptase-inhibitor inhibits human tryptase in a concentration-dependent fashion. Using the tripeptide-nitroanilid tos-Gly-Pro-Arg-pNa as a substrate, a maximal inhibition of 50 % was observed (Fig. 9). Thus, most likely due to steric hindrance, the inhibitor blocks only two of the
30 four catalytic subunits of the tryptase tetramer, leaving the other two subunits accessible to the small substrate. The interaction of the inhibitor with the first two tryptase-subunits can be described mathematically as a tight binding inhibition with a K_i of ~1.4 nM for the complex. The leech-
35 derived tryptase-inhibitor is highly specific and inhibits only trypsin and chymotrypsin with affinities similar to that for tryptase (Tab. 5). In contrast, the K_i -values for the complexes with other proteinases are at least 200 times higher.



Table 5: Specificity of the Leech-derived tryptase inhibitor. ¹⁾ at 0.2 μ M; ²⁾ K_i for the inhibition of two of the four subunits of the tryptase-tetramer; ni, no inhibition at 0.2 μ M; nd, not determined)

Enzym	Substrate	Buffer	Enzym-concentration [nM]	Substrate-concentration [mM]	Pre-incubation	K_m [mM]	K_i app [nM]	K_i [nM]
Tryptase (human)	Tos-Gly-Pro-Arg-pNA	50 mM Tris/HCl pH 7.6 150 mM NaCl, 0.1 % BSA 50 μ g/ml BLH, 0.5% DMSO	0.59	0.1	25 min, 37°C	0.38	1.8	1.4 ²⁾
Trypsin (bovine)	Tos-Gly-Pro-Arg-pNA	50 mM Tris/HCl pH 7.8 0.1 % Triton, 1 % DMSO	0.45	0.1	20 min, 37°C	0.02	4.7	0.9
Chymotrypsin (bovine)	Suc-Val-Pro-Phe-pNA	100 mM Tris/HCl pH 7.8 0.1 % Triton, 1 % DMSO	1.1	0.25	15 min, 37°C	0.04	137	20
Plasmin (human)	Tos-Gly-Pro-Lys-pNA	50 mM Tris/HCl pH 7.8 0.1 % Triton, 1% DMSO	2.7	0.14	30 min, 37°C	nd	30% inhibition ¹⁾	nd
Tissue-Kallikrein (porcine)	D-Val-Leu-Arg-pNA	100 mM Tris/HCl pH 8.2 0.1% Triton	31	0.12	30 min, 25°C	nd	14% inhibition ¹⁾	nd
Thrombin (human)	Tos-Gly-Pro-Arg-pNA	100 mM Tris/HCl pH 8.2 0.1 % Triton, 7 % DMSO	1.8	0.14	30 min, 25°C	nd	12% inhibition ¹⁾	nd
Cathepsin G (human)	Suc-Val-Pro-Phe-pNA	100 mM Tris/HCl pH 7.5 500 mM NaCl 0.1 % Triton, 1 % DMSO	16	0.25	30 min, 25°C	nd	11% inhibition ¹⁾	nd
Plasma-Kallikrein (human)	D-Pro-Phe-Arg-pNA	50 mM Tris/HCl pH 7.8 2 % DMSO	<< 87	0.2	30 min, 37°C	nd	ni ¹⁾	
Faktor Xa (human)	Bz-Ile-Glu-Gly-Arg-pNA	100 mM Tris/HCl pH 8.2 0.1 % Triton, 4%DMSO	10	0.8	30 min, 25°C	nd	ni ¹⁾	
Pankreatic Elastase (porcine)	Suc-Ala-Ala-Ala-pNA	100 mM Tris/HCl pH 7.5 500 mM NaCl 0.1 % Triton, 2%DMSO	72	2	30 min, 25°C	nd	ni ¹⁾	
Neutr. Elastase (human)	MeO-Suc-Ala-Ala-Pro-Val-pNA	100 mM Tris/HCl pH 7.5 500 mM NaCl 0.1%Triton, 2%DMSO	4.2	1	30 min, 25°C	nd	ni ¹⁾	
Urokinase (human)	Pyr-Gly-Arg-pNA	100 mM Tris/HCl pH 8.2 0.1 % Triton, 2 % DMSO	34	0.9	30 min, 25°C	nd	ni ¹⁾	

Example 3: Biologic characterisation

To determine whether the leech-derived tryptase inhibitor affects the cleavage of a biologically relevant substrate by tryptase, its effect on the breakdown of vasoactive intestinal peptide (VIP) was measured. At a concentration of $4 \times 10^{-7} \text{M}$, the inhibitor reduced the breakdown of VIP by 66% (Fig. 10). Thus, the inhibitor blocks not only the tryptase-induced cleavage of the peptide nitroanilid substrate tos-Gly-Pro-Arg-pNA (see Example 2), but also that of a biologically relevant substrate.

Tryptase not only cleaves soluble proteins, but also directly interacts with cells activating cellular functions such as the growth of fibroblasts and keratinocytes. To determine whether the leech-derived tryptase inhibitor blocks these cellular effects of tryptase, its effect on the tryptase-induced growth of cultured human keratinocytes was studied. In the absence of the inhibitor, tryptase (10^{-9}M) markedly stimulated the growth of keratinocytes, increasing their ^3H -thymidin incorporation to $182 \pm 6\%$ of the control. The leech-derived tryptase inhibitor did not significantly affect the baseline growth suggesting a lack of cytotoxic effects (Tab. 6).

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Table 6: Effect of the leech-derived tryptase inhibitor on the proliferation of the human keratinocyte cell line HaCaT.

Condition	Growth rate (% of control)
Medium alone	100
+ Inhibitor $10^{-7}M$	96 ± 3
+ Tryptase	182 ± 6
+ Tryptase + Inhibitor $10^{-7}M$	115 ± 6
+ Tryptase + Inhibitor $10^{-8}M$	120 ± 2

15 Growth rates were calculated as the incorporation of 3H -thymidine in the presence of the agonist and/or inhibitor expressed as a percentage of the incorporation in medium alone. Data are given as mean $\pm$ SEM, $n \geq 2$.

20 The inhibitor greatly reduces the tryptase-induced ($10^{-9}M$) cell growth without significant effect on the proliferation under baseline conditions. Thus, the inhibitor nearly completely blocks of the biologic effect of tryptase without cytotoxic side effect.

25 However, the inhibitor significantly reduced the tryptase-induced proliferation, reducing the 3H -thymidin incorporation to $115 \pm 5\%$ and $120 \pm 2\%$ of the control at a concentration of $10^{-7}M$ and $10^{-8}M$, respectively. As this 3H -thymidin incorporation is
30 similar to that caused by $10^{-11}M$ tryptase ($118 \pm 4\%$), the data suggest that the inhibitor blocks the cellular effect of tryptase by approximately 99%.

Finally, the influence of the leech-derived tryptase inhibitor
35 on the prothrombin time (according to Quick) and the partial



thromboplastin time were measured to determine whether it interferes with the blood coagulation. At a concentration of 10^{-7} M, the inhibitor has no significant effect on both parameters (Tab. 7). Thus, the leech-derived tryptase inhibitor does not significantly inhibit any of the proteases involved in the blood coagulation cascade.

Table 7: Effect of the leech-derived tryptase inhibitor on the blood coagulation.

	Quick	Partial Thromboplastin Time
Control	89%	37,3 sec
Tryptase-Inhibitor	91%	37,1 sec

The inhibitor (100 nM) does not affect the prothrombin time according to Quick and the partial thromboplastin time, demonstrating that the enzymes involved in the coagulation cascade are not inhibited.

Example 4: Pharmaceutical preparation containing the tryptase inhibitor for parenteral administration

A solution prepared in accordance with Example 1 is dialysed against a 0.9 % strength NaCl solution. The concentration of the solution is then adjusted to 1 mg/ml or 10 mg/ml by concentration or by dilution with the same NaCl solution. These solutions are sterilised by ultrafiltration (membranes having 0.22 μ m pores).

The sterilised solutions can be used for intravenous administration.

Example 5: Preparation of Recombinant Tryptase Inhibitor

5.1. Materials

All chemicals used were obtained from Sigma, St. Louis, USA;

Merck, Darmstadt, FRG; Serva, Heidelberg, FRG; Biomol, Hamburg, FRG; Roth, Karlsruhe, FRG; Braun, Melsungen, FRG; Dianova, Hamburg, FRG; Promega, Madison, USA. Restriction endonucleases and DNA-modifying enzymes were purchased from Boehringer, Mannheim, FRG; New England Biolabs, Beverly, USA and Pharmacia-Biotech, Freiburg, FRG. Adenosine-5'- α [35 S]-thiotriphosphate was obtained from Amersham Buchler, Braunschweig, FRG.

Bacto-tryptone, Bacto-peptone, Bacto yeast nitrogen base (without amino acids, w/o), Bacto yeast extract and Bacto-agar were from Difco, Augsburg, FRG. As culture media we used 2xYT [Sambrook et al, 1989]; YPD (10g Bacto yeast extract, 20g Bacto peptone, 20g glucose, pH 6.0); YED (20g Bacto yeast extract, 20g glucose, 6.7g NaH_2PO_4 , pH 6.0) and SD+ (6.7g Bacto nitrogen base (w/o), 20g glucose, 6.7g NaH_2PO_4 , 19mg L-leucine, pH 6.0).

Oligonucleotides were purchased from MWG-Biotech, München, FRG or synthesized by Dr. S. Modrow, München, FRG.

Vectors and strains: The *E. coli* pUC cloning vector was from Pharmacia Biotech Europe GmbH, Freiburg. The *E. coli* - *S. cerevisiae* shuttle and expression vector pVT102U/ α and the yeast strain S-78 were kindly provided by T. Vernet, Montreal, CAN and by C.-W. Chi and Y.-S. Zhang both Shanghai, China [Lit. Vernet et al, Chen et al). *E. coli* TG1 ((lac-pro), supE, thi, hsdD5/F'traD36, proA⁺B⁺, lacI^q, lacZM15) was from Amersham-Buchler, Braunschweig, FRG,; *E. coli* JM105 (thi, rspL, endA, sbcB15, hspR4, (lac-proAB) F'traAB proAB, lacI^q, lacZM15); and *E. coli* HB101 (F⁻, pro⁻, leu⁻, thi⁻ lacY, Sm^r, endoI⁻, recA⁻, r_k⁻, m_k⁻) were from Deutsche Stammsammlung Braunschweig, FRG.

The standard techniques of molecular cloning were performed according to Sambrook et al. [Sambrook et al., 1989] and to M.-D. Rose et al. [Rose et al., 1990].

5.2. Standard analytical methods

a) SDS-PAGE and isoelectric focussing (IEF)

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SDS-PAGES of the proteins were performed with 15-25% polyacrylamide gels following the procedure of Laemmli [Laemmli, 1970]. The gels were either self-prepared and run in a conventional apparatus or in the PhastSystem (Pharmacia, Sollentuna, Sweden). Isoelectric focussing was also done with the PhastSystem using the isoelectric focussing calibration kit pH 3-10.

b) HPLC analysis, amino acid sequencing

Usually 2-3 nmol of protein were analysed by reversed phase-HPLC as detailed previously [Auerswald et al., 1991]. The N-termini were sequenced with a gas-phase sequencer 473A (Applied Biosystems GmbH, Weiterstadt, FRG) following the instructions of the manufacturer.

c) Determination of the protein concentrations

To determine the protein concentration the Pierce BCA* Protein Assay with BSA as standard protein [Smith et al., 1985] was used. $A_{280\text{nm}}$ (1%) was calculated for recombinant LDIT-C using the A_{280} values for aromatic residues and cystines of Mach et al. [1992]: A_{280} (1%) = 3.46, and for protein mixtures A_{280} (1%) = 1.

d) Trypsin inhibition assay

The concentration of inhibitorily active material and the specific inhibitory activity of rLDIT-C was determined indirectly by measuring the residual trypsin activity using the following conditions described by Chase and Shaw, 1970. Test buffer: 0.05M Tris-HCl pH7.6, 150 mM NaCl. 0.1% (v/v) Triton X-100, 600pM trypsin; 100 μ M Tos-Gly-Pro-Arg-p-NA.

e) Determination of K_i values

Equilibrium dissociation constants (K_i) for the complexes of rLDIT-C with trypsin and tryptase were determined essentially as described previously [Bieth, 1980].

5.3. Construction of the synthetic LDIT-C gene.

A synthetic gene coding for a recombinant homologue of LDIT



form C was designed and constructed as outlined in Fig. 11. The DNA sequence was selected on the basis of the amino acid sequence of natural trypsin inhibitor by assistance of the GCG sequence analysis software [UWGCG, Devereux et al., 1984] with the *E.coli* and *S.cerevisiae* codon usages for strongly expressed genes [Bennetzen and Hall, 1982].

The 5'-OH ends of the internal oligonucleotides were phosphorylated using T4 polynucleotide kinase before hybridisation. All six oligonucleotides, 200 pmol each, were heated 5 minutes to 95°C. Hybridization was achieved during cooling down to room temperature within 8 hours. After phenol extraction and ethanol precipitation, internal nicks were ligated by T4 ligase (Boehringer), according to the manufacturers protocol. The material was separated by gel electrophoresis on a low melting agarose (5%) and a 149 bp long fragment was purified using the MERMAID isolation kit from Dianova, Hamburg.

5.4. Construction of cloning vector PRM3.1.10.

The DNA fragment obtained according to 5.3. was ligated into vector pUC18 cut with *EcoRI*/*HindIII* (molar ratio of vector : fragment, 1: 20). Competent *E.coli* TG1 cells were transformed with the ligation mixture and recombinant clones were selected. DNA sequencing was performed using the M13/pUC (-40) sequencing primer, a 17mer, and the reverse sequencing primer (-48), a 24 mer. Vector PRM3.1.10 (Fig. 15) containing the designed sequences of rLDTI-C was used for further experiments.

30

5.5. In vitro production and cytoplasmic expression in *E. coli*

a) The synthetic LDTI-C gene and the expression vector pASK 40 [Skerra et al., 1991] were cleaved separately with *EcoRI* and *HindIII*, purified and ligated. Modified pASK 40 was designated PRM4.1.4 (Fig. 16). The PRM4.1.4 DNA was analysed by an *E.coli* S-30 coupled in vitro transcription translation system, from Promega with S-35 cysteine following the instructions of the



manufacturers. The in vitro transcription translation of vector pRM4.1.4 with a commercially available S-30 *E.coli* lysate showed two major radioactive labelled protein bands with app. MW of 7 kDa and 5 kDa (data not shown). The other strong band detected, seems to be β -lactamase (app. MW of 31 kDa). The 7 kDa protein band is interpreted as the uncleaved fusion protein containing the ompA signal sequence and LDTI-C (theoret. MW 7038 Da) whereas the 5 kDa protein (theoret. MW 5015 Da) band seems to be the cleaved and expected [ANS] LDTI-C which is prolonged by three amino acid residues.

b) For cytoplasmic expression the synthetic LDTI-C gene was ligated after a fill-in reaction into pGEX-3X (Pharmacia) cleaved with SmaI. The resulting vector was named pRM 11.1.4 (Fig.17) and the resulting host strain is *E.coli* 1314. Cytoplasmic glutathione-S-transferase-LDTI-C fusion proteins were found as insoluble inclusion bodies, with *E.coli*. 1314 (HB 101 with pRM11.1.4, data not shown).

5.6. Construction of expression vector pRM 9.1.4

For the expression experiments with yeast the modified alpha mating secretion system pVT102U/ α [Vernet et al., 1987] was selected in which *Trichosanthes* trypsin inhibitor, a small serine proteinase inhibitor of the squash family was expressed successfully [Chen et al., 1992]. Within this system the recombinant inhibitor was correctly folded, cleaved from the signal sequence, protected from proteolytic degradation and it could be purified in two or three steps from yeast fermentation broth.

In order to use the shuttle vector pVT102U/ α , the rLDTI-C gene had to be modified first. The LDTI-C gene (Fig. 11) was mutated by substituting the *EcoRI*/*SphI* cassette with a *XbaI*/*SphI* linker cassette. The sequence of this *XbaI*/*SphI* linker is CTAGATAAAAGAAAGAAGGTTTGC GCATGV. It codes for the C-terminal end (Fig. 11 c) of the alpha mating type signal sequence containing a cleavage site for the KEX2 signal peptidase (Lys Arg) and



the N-terminus of LDTI. The modified LDTI-C gene was assembled via a three fragment ligation using the *XbaI/SphI* linker cassette, the *SphI/HindIII* LDTI-C fragment and the *XbaI/HindIII* cleaved pUC18 vector (molar ratio 10:5:1). After transformation of *E.coli* TG1 recombinant clones were screened by restriction analysis and DNA sequencing using the M13/pUC (-40) primer (Biolabs) and the M13/pUC/-48) reverse primer (Biolabs) (CGCAGTAGCGGTAAACG). The new vector pRM 5.1.5 (Fig. 12a) carried the expected sequence and the *XbaI/HindIII* fragment including the rLDTI-C gene was ligated into *XbaI/HindIII* cleaved pVT102U/ α . The resulting expression vector pRM 9.1.4 (Fig.12b) was isolated and used to transform *S.cerevisiae* strain S-78 according to the method of Becker and Guarante [Becker and Guarante, 1991].

5.7. Expression in *Saccharomyces cerevisiae*

Analytical rLDTI-C expression experiments using yeast strain H005 (S-78 with pRM9.1.4) were carried out with Fernbach flasks (180 - 220 rpm, 28 °C ; pre-culture for 3 days with 100 ml SD(+) media and main cultures for 4 days with 900 ml fresh YED-media). At each day cell density (OD₇₀₀) was determined, pH was adjusted to 6.0 with 1M NaOH, 10 ml yeast extract stock solution 50% and 30 ml 50% (w/v) glucose were added and the inhibition of trypsin was determined.

After transformation of competent S-78 strains with pRM9.1.4 expression of rLDTI was detected. The broth of cultivated recombinant yeast cells showed remarkable trypsin inhibition. The concentrated supernatant gave a protein pattern with the strongest band migrating at an app. MW of 5000 Da after SDS-PAGE (see Fig. 13, lane 2).

The recombinant material was isolated preparatively from culture broth of *S. cerevisiae* cultivated in 1 liter shaker-flasks for 96h. After this time the growth curve of yeast cells reached an OD₇₀₀ of 22.0. Trypsin inhibitory activity was detected after two days and increased parallel to the biomass.

The yeast broth was harvested (6000 g, 20 min, 4 °C) after 96h cultivation and the supernatant was filtered, first through a 0,16 µm membrane and then through a crossflow membrane with a 3 kDa cut-off (Filtron Omega Minisette, Filtron, Karlstein, FRG)). The buffer was exchanged by diafiltration to 20 mM NaH₂PO₄ pH 8.2. The material was purified by cation-exchange chromatography (Fractogel EMD SO₃⁻ 650 (S) column 150-10; Merck), flow rate 3 ml/min, elution buffer 20 mM NaH₂PO₄, pH8.2, 500 mM NaCl.

The data of a representative purification are summarized in Table 8.

Table 8: Results of a typical purification of r LDTI form-C from *Saccharomyces cerevisiae* culture supernatant

Purification step	Volume	Total protein	Active material	Specific activity	Yield
	(ml)	(mg)	(mg)	(%)	(%)
culture supernatant	1000	5620	10.6	0.1	100
culture supernatant, 0,16 µm filtration	965	4600	5.2	0.1	49
retentate, 3K- membrane	125	850	4.6	0.5	43
Fractogel EMD SO ₃ ⁻ main fractions	21	5	3,2	59	30

Total protein was estimated applying the Pierce assay (bovine serum albumin as standard); active material was calculated from trypsin inhibitory assays; yield is given as percentage of isolated material;



From one liter fermentation broth 3 mg rLDTI-C were obtained. The SDS-PAGE of this material showed a homogeneous but relatively broad band migrating at an app. MW of 5000 Da (Fig. 13, lane 3). About 85 % of rLDTI-C eluted as a sharp peak at 28% acetonitrile when analysed by RP18 HPLC. The amino acid sequencing of peak 1 (Fig. 14) revealed the expected N-terminus KKVCACPK. But small heterogeneities were observed after RP-HPLC and a different N-terminus was identified (peak 2) starting with 11 additional amino acids of C-terminal part of alpha factor signal peptide (Fig. 14).

This demonstrates that the endogeneous KEX 2 protease of yeast did cleave with high accuracy after LysArg, the recognition site of the signal peptidase KEX2, and still in front of the two N-terminal amino acid residues LysLys of LDTI. Isoelectric focussing with the PhastSystem demonstrated that the isoelectric point of rLDTI was above pH 10.

The determined inhibition constants of the complexes trypsin-LDTI-C and trypsin-LDTI-C are similar to those with natural LDTI. The measured specific trypsin inhibitory activity of 60 % is comparable to other recombinant inhibitors.

rLDTI-C inhibits human trypsin in a fashion similar to the naturally occurring leech-derived trypsin inhibitor: using the tripeptide-nitroanilid Tos-Gly-Pro-Arg-pNa as a substrate, a maximal inhibition of ~50% was observed, and a K_i of 1.9 nM was calculated for the complex between trypsin and rLDTI-C.

Example 6: Construction of pFBY166

pFBY166 is a pUC18 derived plasmid that contains a 1085bp BamHI fragment. This fragment contains the CUP1 promoter fused to the ATG of the α -factor leader, a stuffer fragment and the α -factor terminator. The precise way the fusions were engineered enable the insertion of ORF (open reading frame) containing fragments either at the ATG by using the EcoRI site, after the signal sequence by using a PstI site or after the α -factor leader



sequence by insertion after the *Bgl*III site. The ORF to be expressed should ideally have a *Sal*I site at their 3' end to facilitate fusion to the terminator that is preceded by a *Sal*I site, and have no *Bam*HI sites within their sequence, as cleavage of this plasmid at the two *Bam*HI sites excises the whole expression cassette so that it can easily be cloned into a yeast shuttle vector.

pFBY166 contains a 425 bp *Bam*HI/*Eco*RI fragment of the CUP1 promoter, corresponding to nucleotides 1080 to 1505 of EMBL GENBANK accession number K02204. The CUP1 promoter allows expression in a copper regulated manner.

The ATG is provided as part of the α -1 factor pheromone signal sequence and leader, nucleotides 293 to 527 of the EMBL GENBANK accession number X01581 followed by the sequence, AGATCTTGC, which positions a *Bgl*III site, which is unique in pFBY139, just before the normal position for the LysArg KEX2 cleavage site. If fusions are required to just a signal sequence this can be achieved by using the unique *Pst*I site which is present within the region encoding the signal sequence. The *Bgl*III site is followed by a sequence of no importance as it is always removed when the incoming ORF is cloned into the plasmid between either the *Eco*RI, *Pst*I or *Bgl*III sites and the *Sal*I site which marks the end of the stuffer fragment and the beginning of the α -1 factor pheromone terminator sequences, nucleotides 825 to 1100 of EMBL GENBANK accession number X01581. This is followed immediately by the sequence AATTCGGATCC which encodes the *Bam*HI site that bounds this end of the expression cassette.

This plasmid can be constructed using polymerase chain reaction (PCR) fragments from yeast genomic DNA.

All oligonucleotides used in the PCR reaction are synthesized using an automatic DNA synthesizer. The PCR reactions are carried out in a PCR unit from Perkin Elmer under the following conditions:



20 mM of the oligonucleotides in question are incubated in 0.1 ml buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂) with 2.5 units of Taq DNA-polymerase and 0.2 mM of dATP, dCTP, dTTP and dGTP. The reactions are incubated for 30 cycles: 30 sec at 92°C, for 1 min at 42°C and at 72°C for 1 min.

The fragment comprising most of the α -factor signal and leader sequences is generated from genomic yeast DNA using the PCR fragments 1 and 2:

1. 5' GTGCGAATTCAAAATGAGATTTCCTTCAATTTTACTGCAG 3'
2. 5' CAAAGTCGACTTTATCCAGCAAGATCTCTTCTTCTTTAGCAGCAATGC 3'

The fragment comprising the α -factor terminator is generated from genomic yeast DNA using the PCR fragments 3 and 4:

3. 5' GAAGAGATCTTGCTGGATAAAGTCGACTTTGTTCCCACTGTACTTTTAGC 3'
4. 5' CCGGGGATCCGAATTAATTCTCTTAGGATTCG 3'

The fragment comprising the CUP1 promoter is generated from genomic yeast DNA using the PCR fragments 5 and 6:

5. 5' TAGAGGATCCCCATTACCGACATTTGGGCGCTATACGTGC 3'
6. 5' CGACGAATTCACAGTTTGTTTTCTTAATATCTATTTCG 3'

and subsequent cleavage with *Bam*HI and *Eco*RI.

The fragment comprising most of the α -factor signal and leader sequences and the fragment comprising the α -factor terminator are mixed and reamplified in a PCR reaction with oligonucleotide 1 and oligonucleotide 3 and cut with *Eco*RI and *Bam*HI. The later amplified fragment and the fragment comprising the CUP1 promoter are cloned into pTZ18R cut with *Bam*HI and treated with bacterial alkaline phosphatase to create pFBY139.



**Example 7: Construction of pHE 174**

Expression of trypsin inhibitor under control of the regulated CUP1 promoter

- 5 A synthetic gene encoding trypsin inhibitor in preferred yeast codon usage is assembled from 3 synthetic oligonucleotides in a PCR reaction. In addition, the gene is extended at its 5' end to provide for convenient in-frame fusion to the α -factor leader in plasmid pFBY 166.

10

The following 3 oligonucleotides are synthesized using an automatic DNA synthesizer:

1. 5'-AAAGATCTTG CTGGATAAAA GAAAGAAGGT TTGCGCCTGT CCAAAGATTT
15 TGAAGCCAGT TTGTGGTTCT GACGGTCGTA CC-3'
2. 5'-ACAAGAACT TCAGACTTAA TAGAAACACC GTTACAACGG GCAATACAAG
AGTTGGCGTA GGTACGACCG TCAGAACCAC-3'
- 20 3. 5'-TTGTCGACTC AGTTCAAAAT ACCGGTTGGA CAAGAACCTT CAGACTTAA-
3'

25

Of these 3 oligonucleotides a 170bp fragment is assembled in the following polymerase chain reaction (PCR) using the PCR unit from Perkin Elmer and the following conditions:

30

20 mM of oligonucleotides 1 and 3 and 20 nM of oligonucleotide 2 are incubated in 0.1 ml buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂) with 2.5 units of Taq DNA-polymerase and 0.2 mM of dATP, dCTP, dTTP and dGTP. The reaction is incubated for 30 cycles: 30 sec at 92°C, for 1 min at 42°C and at 72°C for 1 min.

35

The 170 bp PCR fragment is isolated over a 2% agarose gel, restricted with *Bgl*II and *Sal*I and ligated into *Bgl*II and *Sal*I cut pFBY 166 (supra). *E. coli* HB101 is transformed with the resulting plasmid pHE174.

The transformed *E. coli* strain is designated *E. coli*/pHE174.

Correct fusion of the PCR fragment to the α -factor leader and correct sequence of the tryptase inhibitor ORF is confirmed by sequencing.

5 **Example 8: Construction of pHE 175 and 175R**
 2 micron vectors with the tryptase inhibitor
 expression cassette

10 For the expression in yeast pDP34 is used as vector. pDP34 (EP-A-340 170, Fig. 3 therein) is a yeast-*E. coli* shuttle vector with the ampicillin resistance marker for *E. coli* and the URA3 and dLEU2 yeast selective markers. It contains the complete 2 micron sequences in the A form and is REP1, REP2 and FLP proficient.

15 Plasmid pDP34 is digested with *Bam*HI and the sticky ends are dephosphorylated by alkaline phosphatase treatment. pHE174 is digested with *Bam*HI and the 1119bp fragment containing the complete tryptase inhibitor expression cassette ligated into
20 *Bam*HI-cut pDP 34. *E. coli* HB 101 is transformed with the resulting plasmids pHE 175 and 175R. Orientation of the insert is tested by digestion with *Sal*I. pHE 175 contains the Tryptase inhibitor expression cassette in a clockwise orientation with respect to dLEU2, pHE 175R in anticlockwise orientation with
25 respect to the dLEU2 marker.

30 **Example 9: Construction of pHE 176**
 The tryptase inhibitor ORF fused to the invertase
 signal sequence (SUC2)

To provide for an alternative secretion system, the tryptase inhibitor ORF is fused to the signal sequence of the yeast invertase gene SUC2.

35 The 2 following oligonucleotides are made:

1. 5'-TTGTCGACTC AGTTCAAAT A-3'

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2. 5'-AAGAATTCAT GCTTTTGCAA GCTTTCCTTT TCCTTTTGGC TGGTTTTCGA
GCCAAAATAT CTGCAAAGAA GGTTCGCGCC TGTC-3'

pHE 174 is used as template DNA for a polymerase chain reaction
as described in example 7. 20 ng of template pHE 174 is
incubated with 20 mM of the oligonucleotide primers under the
experimental conditions as in example 7.

The 214bp amplified PCR fragment is isolated over a 2% agarose
gel, restricted with *EcoRI* and *SalI* and ligated into *EcoRI* and
SalI cut vector pFBY 166.

E. coli HB 101 is transformed with the resulting plasmid pHE
176. Correct sequence of the SUC2 signal sequence-tryptase
inhibitor fusion is confirmed by sequencing.

Example 10: Construction of pHE 177 and pHE 177R
2 micron vectors with the tryptase inhibitor
expression cassette with the SUC2 signal
sequence

In analogy to example 8, the 918 bp *BamHI* fragment containing
the tryptase inhibitor expression cassette is excised from pHE
176 by *BamHI* digestion and inserted into *BamHI* cut pDP 34. *E.*
coli HB 101 is transformed with the resulting plasmids pHE 177
and pHE 177R. Orientation of the insert is tested by digestion
with *SalI*. pHE 177 contains the tryptase inhibitor expression
cassette in a clockwise orientation with respect to dLEU2, pHE
177R in an anticlockwise orientation.

Example 11: Construction of *Saccharomyces cerevisiae* strain
TR 1456

Saccharomyces cerevisiae strain TR1456 is constructed as
disclosed in EP-A-341 215. Starting with *Saccharomyces*
cerevisiae strain H449 (DSM 4413, MATa, leu23,112, ura3, prb1
[*cir*⁰]), in two subsequent series of experiments the two
carboxypeptidases *yscA* and *yscY* are removed from strain H449 by

disruption of their encoding genes KEX1 and PRC1, respectively. First, the gene encoding ysca, KEX1, is disrupted.

For this purpose, strain H449 is transformed with a DNA
 5 fragment encoding the KEX1 gene, with the full URA3 gene
 inserted in the middle of the KEX1 coding region. Uracil proto-
 trophic transformants are selected and tested for the absence
 of ysca activity. Next, the URA3 gene inserted at the KEX1
 locus is disrupted by transformation with a plasmid containing
 10 a disrupted version of the gene, URA3Δ5 (see EP-A-341 215).
 Transformants which are uracil auxotrophic are selected and in
 the following step disrupted in their endogenous PRC1 gene
 coding for the carboxypeptidase yscY. The experiment is carried
 out in a totally analogous manner as described for the
 15 disruption of KEX1. The finally resulting isogenic derivative
 of strain H449 is called TR1456 and has the following genotype:

TR1456 = MATa, leu2-3, 112, ura3, prb1, kex1::ura3, prc1::ura3,
 [cir^o]

20 Example 12: Transformation of strain TR 1456 with plasmids
 pHE 175, 175R, 177 and 177R

The plasmids pHE 175, 175R, 177 and 177R are introduced into
 25 the host strains H449 and TR1456, resp., using the
 transformation protocol described by Hinnen et al. (Proc. Natl.
 Acad. Sci. USA (1978), 75, 1929). Further details of the
 procedure are as described in EP-A-341 215. Transformed yeast
 cells are selected on yeast minimal medium, supplemented with
 30 leucine and lacking uracil. Single transformed yeast clones are
 isolated and referred to as:

Saccharomyces cerevisiae TR 1456 / pHE 175
Saccharomyces cerevisiae TR 1456 / pHE 175R
Saccharomyces cerevisiae TR 1456 / pHE 177
 35 *Saccharomyces cerevisiae* TR 1456 / pHE 177R
Saccharomyces cerevisiae H449 / pHE 175
Saccharomyces cerevisiae H449 / pHE 175R
Saccharomyces cerevisiae H449 / pHE 177

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Saccharomyces cerevisiae H449 / pHE 177R

Example 13: Secretion of leech-derived tryptase inhibitor by
TR 1456 transformed with plasmid pHE 177

5

Cells of *Saccharomyces cerevisiae* TR 1456 / pHE 177 are grown
in two subsequent precultures of 20 ml each. The synthetic
medium is composed of:

10	6.7	g/l	Difco Yeast Nitrogen Base (without amino acids)
	10	g/l	L-asparagine
	1	g/l	L-histidine
	20	g/l	glucose
15	0.02	g/l	L-leucine

The pH of the medium is adjusted to 5.8. The first preculture
is grown for 60 h at 28°C and 180 r.p.m. The second preculture
is inoculated with 2% (v/v) of the first preculture and in-
cubated for 24 h at 28 °C and 180 r.p.m.

The medium of the main culture is composed of:

	5	g/l	peptone
25	10	g/l	yeast extract
	20	g/l	glucose
	40	g/l	sucrose
	3	g/l	ammonium sulfate
	2	g/l	potassium dihydrogenphosphate
30	0.5	g/l	magnesium sulfate heptahydrate
	0.1	g/l	sodium chloride
	0.1	g/l	calcium chloride
	10 ⁻⁵	g/l	biotin

35 The main culture (100 ml medium) is inoculated with about 10⁶
cells/ml and incubated for 72 h at 28°C and 180 r.p.m..



Immediately following the inoculation, sterile copper sulfate is added at a concentration of 1 mM to the culture.

At the end of the fermentation, aliquots of the culture are taken, the cells are removed by centrifugation and the culture supernatant is analyzed for activity of the leech-derived tryptase inhibitor by titration of the inhibitor with trypsin as described under 2.2. i).

10 Example 14: Analytics of the leech-derived tryptase inhibitor from fermentation cultures of *Saccharomyces cerevisiae* strain TR 1456 / pHE177 using reversed phase HPLC

15 Samples from culture supernatants of strain TR 1456 / pHE177 are subjected to HPLC analysis under the following conditions:

A Merck Lichrospher 1000 RP-8 column (4x250mm, 10um) is used. Mobile phase A is made from water (Nanopur®, Barnstead) containing 0.1% (v/v) trifluoroacetic acid. Mobile phase B is made from 20% water (Nanopur®, Barnstead) and 80% of acetonitrile (HPLC-grade, Fluka) containing 0.09% (v/v) of trifluoroacetic acid.

25 Chromatographic separations are performed at a flow rate of 1.5ml/min running the following gradient (Table 9). The eluents are monitored by absorbance at 214nm.

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Table 9:

t (min)	%A	%B
0	85	15
8	85	15
28	65	35
34	0	100
37	0	100
42	85	15
46	85	15

One major peak with a retention time of 19.05 min is observed on the chromatogram that is present in strains bearing the inhibitor expression plasmid, but absent in untransformed strains. Further analysis revealed that this peak contains one species of the leech-derived tryptase inhibitor with an apparent Mr of 4738,8 as detected by mass spectroscopy. This value is in good agreement with the calculated Mr value of 4738, pointing to the full-length inhibitor molecule carrying both, the correct N-terminus and C-terminus.

The chemical molecular weight of the inhibitor is determined by matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) using a home-built instrument (Boernsen et al., Chimica (1990) 44, 412-416).

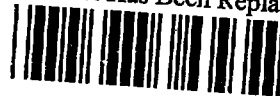
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SEQUENCE LISTING

GENERAL INFORMATION:

- (i) APPLICANT:
 (A) NAME: UCP Gen-Pharma AG
 (B) STREET: Kraftstr. 6
 (C) CITY: Zuerich
 (E) COUNTRY: Switzerland
 (F) POSTAL CODE (ZIP): CH-8044
 (G) TELEPHONE: 01 251 10 60
- (ii) TITLE OF INVENTION: Tryptase Inhibitor
- (iii) NUMBER OF SEQUENCES: 9
- (iv) COMPUTER READABLE FORM:
 (A) MEDIUM TYPE: Floppy disk
 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

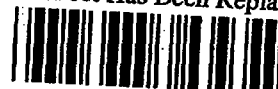
INFORMATION FOR SEQ ID NO: 1:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 42 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: NO
- (vi) ORIGINAL SOURCE:
 (A) ORGANISM: *Hirudo medicinalis*
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:
- | | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Lys | Lys | Val | Cys | Ala | Cys | Pro | Lys | Ile | Leu | Lys | Pro | Val | Cys | Gly | Ser |
| 1 | | | | 5 | | | | | 10 | | | | | 15 | |
| Asp | Gly | Arg | Thr | Tyr | Ala | Asn | Ser | Cys | Ile | Ala | Arg | Cys | Asn | Gly | Val |
| | | 20 | | | | | | 25 | | | | | 30 | | |
| Ser | Ile | Lys | Ser | Glu | Gly | Ser | Cys | Pro | Thr | | | | | | |
| | | 35 | | | | | 40 | | | | | | | | |

INFORMATION FOR SEQ ID NO: 2:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 43 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: NO
- (vi) ORIGINAL SOURCE:
 (A) ORGANISM: *Hirudo medicinalis*

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Lys Lys Val Cys Ala Cys Pro Lys Ile Leu Lys Pro Val Cys Gly Ser
 1 5 10 15
 Asp Gly Arg Thr Tyr Ala Asn Ser Cys Ile Ala Arg Cys Asn Gly Val
 20 25 30
 Ser Ile Lys Ser Glu Gly Ser Cys Pro Thr Gly
 35 40

) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 46 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Lys Lys Val Cys Ala Cys Pro Lys Ile Leu Lys Pro Val Cys Gly Ser
 1 5 10 15
 Asp Gly Arg Thr Tyr Ala Asn Ser Cys Ile Ala Arg Cys Asn Gly Val
 20 25 30
 Ser Ile Lys Ser Glu Gly Ser Cys Pro Thr Gly Ile Leu Asn
 35 40 45

INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 126 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: YES

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Hirudo medicinalis*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

ARGTNT GYGCNTGYCC NAARATHYTN AARCCNGTNT GYGGNWSNGA YGGNMGNACN 60
 CNAAYW SNTGYATHGC NMGNTGYAAY GGNGTNWSNA THAARWSNGA RGGNWSNTGY 120
 CN 126

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INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 129 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: YES

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Hirudo medicinalis*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

AARAAGTNT GYGCNTGYCC NAARATHYTN AARCCNGTNT GYGGNWSNGA YGGNMGNACN	60
TAYGCNAAYW SNTGYATHGC NMGNTGYAAY GGNGTNWSNA THAARWSNGA RGGNWSNTGY	120
ACNCGGN	129

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 138 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: YES

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Hirudo medicinalis*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

AARAAGTNT GYGCNTGYCC NAARATHYTN AARCCNGTNT GYGGNWSNGA YGGNMGNACN	60
AYGCNAAYW SNTGYATHGC NMGNTGYAAY GGNGTNWSNA THAARWSNGA RGGNWSNTGY	120
ACNCGGNA THYTNAAY	138

(3) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 149 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: YES

(iii) ANTI-SENSE: NO

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(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Hirudo medicinalis*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

TTTCGAAGA AGGTTTGCGC ATGCCCAAAG ATCTTGAAGC CAGTCTGTGG TTCTGACGGT 60
 GTACATATG CTAACTCATG CATCGCTCGT TGTAACGGTG TATCGATCAA GTCTGAAGGT 120
 CTTGTCCAA CCGGTATTTT AAATAATA 149

) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 149 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: YES

(iii) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Hirudo medicinalis*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TTATTAG TTTAAGTTAC CGGTTGGACA AGAACCTTCA GACTTGATCG ATACACCGTT 60
 ACGAGCG ATGCATGAGT TAGCATATGT ACGACCGTCA GAACCACAGA CTGGCTTCAA 120
 TTTGGG CATGCGCAA CTTCTTCG 149

INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 929 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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- (B) LOCATION: 435..440
- (D) OTHER INFORMATION: /function= "EcoRI site"

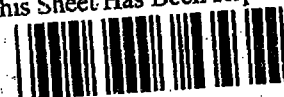
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- (A) NAME/KEY: misc_feature
- (B) LOCATION: 642..647
- (D) OTHER INFORMATION: /function= "Sali site"

(ix) FEATURE:

- (A) NAME/KEY: promoter
- (B) LOCATION: 1..443
- (D) OTHER INFORMATION: /phenotype= "CUP1 promoter"

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(ix) FEATURE:

- (A) NAME/KEY: sig_peptide
- (B) LOCATION: 444..500
- (D) OTHER INFORMATION: /function= "SUC2 invertase signal sequence"

(ix) FEATURE:

- (A) NAME/KEY: mat_peptide
- (B) LOCATION: 501..641
- (D) OTHER INFORMATION: /product= "tryptase inhibitor"

(ix) FEATURE:

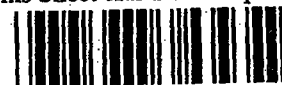
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- (B) LOCATION: 648..923
- (D) OTHER INFORMATION: /standard_name= "alpha-factor terminator"

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 924..929
- (D) OTHER INFORMATION: /function= "BamHI site"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

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AAACACTT TTGTATTATT TTTCCTCATA TATGTGTATA GGTTTATACG GATGATTTAA	120
TATTACTTC ACCACCCTTT ATTTCAAGCT GATATCTTAG CCTTGTTACT AGTTAGAAAA	180
ACATTTTT GCTGTCAGTC ACTGTCAAGA GATTCTTTTG CTGGCATTTC TTCTAGAAGC	240
AAAGAGCG ATGCGTCTTT TCCGCTGAAC CGTTCCAGCA AAAAAGACTA CCAACGCAAT	300
CGATTGTC AGAATCATAT AAAAGAGAAG CAAATAACTC CTTGTCTTGT ATCAATTGCA	360
TAATATC TTCTTGTTAG TGCAATATCA TATAGAAGTC ATCGAAATAG ATATTAAGAA	420
CAAACTG TAACGAATTC AAAATGCTTT TGCAAGCTTT CCTTTTCCTT TTGGCTGGTT	480
CAGCCAA AATATCTGCA AAGAAGGTTT GCGCCTGTCC AAAGATTTTG AAGCCAGTTT	540
CTCTGA CGGTCGTACC TACGCCAACT CTTGTATTGC CCGTTGTAAC GGTGTTTCTA	600
CTCTGA AGGTTCTTGT CCAACCGGTA TTTTGAAGTG AGTCGACTTT GTTCCCACTG	660
TTTAGC TCGTACAAAA TACAATATAC TTTTCATTTT TCCGTAAACA ACATGTTTTT	720
TAATA TCCTTTTCTA TTTTTCGTTC CGTTACCAAC TTTACACATA CTTTATATAG	780
CACTT CTATACACTA AAAAATAAG ACAATTTTAA TTTTGCTGCC TGCCATATTT	840
CGTTA TAAATTCCTA TAATTTATCC TATTAGTAGC TAAAAAAGA TGAATGTGAA	900
CTTA AGAGAATTAA TTCGGATCC	929



M/35058

CLAIMS

5 1. A purified inhibitor molecule of human tryptase being a polypeptide; or the functional equivalents thereof showing tryptase inhibitor activity.

10 2. The tryptase inhibitor of claim 1 obtainable from extracts of leeches.

3. The tryptase inhibitor of claim 1 or 2, characterized by

15 a) inhibiting human tryptase with a K_i value in the range of approximately 0,1 to 10 nM; and

b) leaving the proteinases involved in the human blood coagulation cascade substantially unaffected.

20 4. The tryptase inhibitor of one of claims 1 to 3, essentially characterized by the amino acid sequence:

Lys-Lys-Val-Cys-Ala-Cys-Pro-Lys-Ile-Leu 10

Lys-Pro-Val-Cys-Gly-Ser-Asp-Gly-Arg-Thr 20

Tyr-Ala-Asn-Ser-Cys-Ile-Ala-Arg-Cys-Asn 30

25 Gly-Val-Ser-Ile-Lys-Ser-Glu-Gly-Ser-Cys 40

Pro-Thr-X 42

30 wherein the C-terminal residue X represents H (SEQ ID NO:1), -Gly (SEQ ID NO:2) or -Gly-Ile-Leu-Asn (SEQ ID NO:3);

35 or the functional equivalents thereof, having one or more of the amino acids of the above sequence substituted or deleted, or having one or more amino acids added, without substantially affecting its tryptase inhibitor activity.

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5. A functional equivalent of the inhibitor of one of claims 1 to 4 comprising the amino acid sequence

R¹-Cys-Pro-Lys-Ile-Leu
Lys-Pro-Val-Z-Gly-Ser-Asp-Gly-Arg-Thr
Tyr-Ala-Asn-Ser-Cys-Ile-Ala-R²

wherein

the N-terminal residue R¹ represents Ala- or Cys-Ala-;
the C-terminal residue R² represents -Arg or -Arg-Cys; and
Z defines any amino acid.

6. An inhibitor according to anyone of claims 1 to 5 obtainable by peptide synthesis or recombinant DNA technology.
7. A polynucleotide encoding a polypeptide with tryptase inhibitor activity as defined in anyone of claims 1 to 6; or the complementary polynucleotide thereof.
8. The polynucleotide of claim 7 comprising the nucleotide sequence (SEQ ID NO:4):

1 AARAARGTNTGYGCNTGYCCNAARATHYTNAARCCNGTNTGYGGNWSNGA
51 YGGNMGNACNTAYGCNAAAYWSNTGYATHGCNMGNTGYAAAYGGNGTNWSNA
101 THAARWSNGARGGNWSNTGYCCNACNX

wherein R denotes A or G;
M denotes A or C;
W denotes A or T;
S denotes C or G;
Y denotes C or T;
H denotes A, C, or T;
N denotes any nucleotide
X denotes 3'-OH (SEQ ID NO:4)

GGN (SEQ ID NO:5) or

GGN ATH YTN AAY (SEQ ID NO:6)

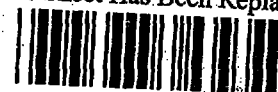




or the complementary strand thereof; and the nucleotide sequences which hybridize to the aforementioned DNA sequence.

- 5
9. The polynucleotide of claim 7 comprising a nucleotide sequence substantially corresponding to nucleotide residues 1 to 149, or preferably 7 to 144, of SEQ ID NO:7; or a fragment thereof.
- 10
10. The polynucleotide of claim 7 comprising a nucleotide sequence substantially corresponding to nucleotide residues 1 to 149, or preferably 10 to 147 of SEQ ID NO:8; or a fragment thereof.
- 15
11. An oligonucleotide which hybridizes to a nucleotide sequence encoding a polypeptide with trypsin inhibitor activity.
- 20
12. The oligonucleotide of claim 11 comprising a nucleotide sequence which is substantially complementary to the nucleotide sequence of residue 22 to 87 of SEQ ID NO:5.
- 25
13. A polynucleotide encoding a polypeptide with trypsin inhibitor activity and obtainable by hybridizing with an oligonucleotide of one of claims 11 and 12.
- 30
14. A polypeptide expression cassette comprising a promoter operably linked to a polypeptide encoding DNA sequence according to one of claims 7 to 10 and 13 and to a DNA sequence containing transcription termination signals.
- 35
15. The expression cassette according to claim 14 comprising a promoter operably linked to a first DNA sequence encoding a signal peptide linked in the proper reading frame to a second polypeptide encoding DNA sequence according to one of claims 7 to 10 and 13, and a DNA sequence containing transcription termination signals.

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16. The expression cassette according to claim 15 wherein the promoter is selected from the group consisting of CUP1p, and GAPDHP.
- 5 17. The expression cassette according to claim 14 or 15, wherein the signal sequence is selected from the group consisting of the α -factor leader, PHO5, and SUC2.
- 10 18. The expression cassette according to one of claims 14 to 17, wherein the terminator is selected from the group consisting of the α -factor terminator and PHO5 terminator.
- 15 19. The expression cassette according to claim 18 comprising the DNA sequence of SEQ ID NO:9 or a functional equivalent thereof.
20. A polypeptide encoded by a polynucleotide of claim 13.
- 20 21. A vector for the transformation of eukaryotic or prokaryotic hosts comprising a polynucleotide of one of the claims 7 to 10 or 13.
- 25 22. A vector for the transformation of eukaryotic or prokaryotic hosts comprising an expression cassette as defined in one of claims 14 to 19.
- 30 23. The vector of claim 21 or 22 which is a two-micron based yeast vector.
- 35 24. The vector of one of claims 21 to 23 selected from
- a) pRM9.1.4 as deposited with the DSM and having the accession number DSM 9271;
 - b) pRM11.1.4 as deposited with the DSM and having the accession number DSM 9272;
 - c) pRM5.1.5 as deposited with the DSM and having the accession number DSM 9270;
 - d) pRM4.1.4 as deposited with the DSM and having the accession number DSM 9269; and





e) pRM3.1.10 as deposited with the DSM and having the accession number DSM 9268.

25. The vector according to one of claims 21 to 23 selected from the group consisting of pHE175, pHE175R, pHE177, and pHE177R.

26. A method of preparing an inhibitor of human tryptase by

a) obtaining a leech extract, and

b) purifying the extract by dialysis and column chromatography.

27. The method of claim 26, wherein

a) the extract is dialyzed against a suitable buffer; and

b) the dialyzed extract is purified by cation exchange chromatography; bio-specific affinity chromatography and a further cation exchange chromatography step.

28. A method of preparing recombinant tryptase inhibitor, which method comprises

a) transforming a prokaryotic or eukaryotic host with a vector according one of to claims 21 to 25;

b) inducing the expression of the tryptase inhibitor encoding sequence;

c) recovering the expression product; and optionally

d) removing from the obtained product peptide fragments not required for tryptase inhibitor activity and/or optionally renaturing the product.

29. A tryptase inhibitor obtainable by a method of claim 28.

30. A pharmaceutical composition comprising a tryptase inhibiting amount of a polypeptide according to anyone of claims 1 to 6, 20 and 29, optionally in combination with a pharmaceutically acceptable carrier or diluent.





31. The use of a tryptase inhibitor of anyone of claims 1 to 6, 20 and 29 in diagnosing functional disorders.
32. The use of a polypeptide according to anyone of claims 1 to 6, 20 and 29 for preparing a pharmaceutical composition for the treatment of mast cell related disorders.
33. The use of claim 31 for preparing a pharmaceutical composition for the treatment of asthma, intestinal lung disease, arthritis, periodontal disease, allergic disorders, skin disorders, psoriasis or blood clotting disorders.
34. A prokaryotic or eukaryotic host transformed with a vector of one of claims 21 to 25, and variants and mutants thereof.
35. A host according to claim 34 which is *Saccharomyces cerevisiae* or *E. coli*.
36. A eukaryotic host of claim 35 deposited with the DSM and having the accession number DSM 9273, and variants and mutants thereof capable of producing a tryptase inhibiting polypeptide.
37. A host according to claim 35 which is selected from the group consisting of
Saccharomyces cerevisiae TR 1456 / pHE 175
Saccharomyces cerevisiae TR 1456 / pHE 175R
Saccharomyces cerevisiae TR 1456 / pHE 177
Saccharomyces cerevisiae TR 1456 / pHE 177R
Saccharomyces cerevisiae H449 / pHE 175
Saccharomyces cerevisiae H449 / pHE 175R
Saccharomyces cerevisiae H449 / pHE 177
Saccharomyces cerevisiae H449 / pHE 177R,
and variants and mutants thereof capable of producing a tryptase inhibiting polypeptide.

