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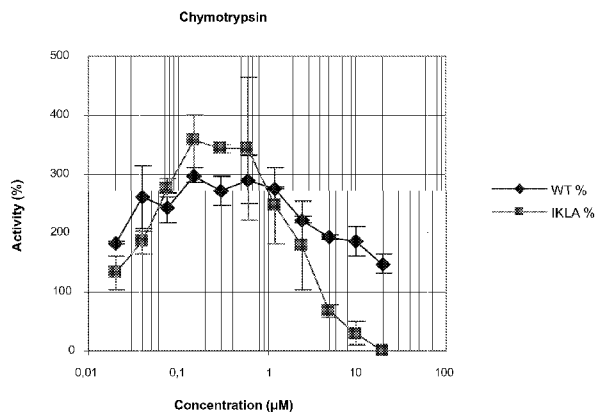
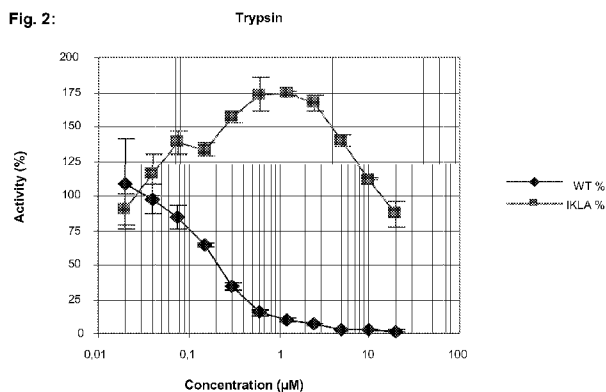
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(54) Title: INSECT INHIBITING PLANT SERPIN MUTANTS



(57) Abstract: The present invention relates to a method to limit insect and/or mite damage in plants, by the use of mutant plant proteinase inhibitors. More specifically, it relates to the use of mutant serpins of which the reactive centre loop has been replaced by an artificial sequence. Preferably, said mutant serpins use an Arabidopsis thaliana serpin-1 backbone, or a homologue thereof. Said mutant serpins have a specificity other than the wild type serpins, and by modulating the reactive loop center, specific inhibitors against specific insect and/or mite proteases can be developed. Those mutant serpins can be used to inhibit or limit insect and/or mite damage, such as the damage caused by insect feeding.

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INSECT INHIBITING PLANT SERPIN MUTANTS

The present invention relates to a method to limit insect and/or mite damage in plants, by the use of mutant plant proteinase inhibitors. More specifically, it relates to the use of mutant serpins of which the reactive centre loop has been replaced by an artificial sequence. Preferably, said mutant serpins use an *Arabidopsis thaliana* serpin-1 backbone, or a homologue thereof. Said mutant serpins have a specificity other than the wild type serpins, and by modulating the reactive loop center, specific inhibitors against specific insect and/or mite proteases can be developed. Those mutant serpins can be used to inhibit or limit insect and/or mite damage, such as the damage caused by insect feeding.

Insect pests are a serious problem in agriculture. They destroy millions of acres of staple crops such as corn, soybeans, peas, and cotton. Farmers must apply millions of liters of synthetic pesticides to combat these pests. However, synthetic pesticides pose many problems. They are expensive, force the emergence of insecticide-resistant pests, and they constitute a serious risk for the environment.

In order to develop a more economic and environmental pest control, biological approaches to pest control have been tried. In some cases, crop growers have introduced natural predators of the species sought to be controlled, such as non-native insects, fungi, and bacteria like *Bacillus thuringiensis*. Alternatively, crop growers have introduced large colonies of sterile insect pests in the hope that mating between the sterilized insects and fecund wild insects would decrease the insect population. However, none of these methods have been really successful.

As mentioned above, certain species of microorganisms of the genus *Bacillus* are known to possess pesticidal activity against a broad range of insect pests including Lepidoptera (Dulmage *et al.*, 1981), Diptera (Goldberg and Margalit, 1977), Coleoptera (Krieg *et al.*, 1987) and Hemiptera (Mathavan *et al.*, 1987). *Bacillus thuringiensis* is probably the most successful biocontrol agents discovered to date. Pesticidal activity appears to be concentrated in parasporal crystalline protein inclusions, although insecticidal proteins have also been isolated from the vegetative growth stage of *Bacillus*. Several genes encoding these insecticidal proteins have been isolated and characterized. As a non limiting example, such genes have been disclosed in WO9116432, WO9421795 and US5366892.

Microbial pesticides, particularly those obtained from *Bacillus* strains, have played an important role in agriculture as alternatives to chemical pest control. Insecticidal proteins isolated from strains of *Bacillus thuringiensis*, known as δ -endotoxins or Cry toxins, are initially produced in an inactive protoxin form. These protoxins are proteolytically converted into an active toxin through the action of proteases in the insect gut.

Once activated, the Cry toxin binds with high affinity to receptors on epithelial cells in the insect gut, thereby creating leakage channels in the cell membrane, lysis of the insect gut, and subsequent insect death through starvation and septicemia.

During recent years, agricultural scientists have developed crop plants with enhanced insect resistance by genetically engineering crop plants to produce insecticidal proteins from *Bacillus*.
5 Such plants have been disclosed in, amongst others, WO9116432 and WO9421795. However, these Bt insecticidal proteins only protect plants from a relatively narrow range of pests. Thus, there is an immediate need for methods that enhance the effects of insecticidal proteins.

One possibility to enhance the insect resistance is the co-expression of the Bt insecticidal proteins with another polypeptide having insecticidal activity, as disclosed in WO2005083095.
10 Alternatively, the application of other broad spectrum insect inhibitory polypeptides, such as proteinase inhibitors has been studied. An overview of proteinase inhibitor genes used in combat against insects, pest and pathogens is given by Haq *et al.* (2004). However, the pH dependency and specificity of the proteinase inhibitor is extremely important and not all proteinases inhibitors
15 are successful. In some cases, the transgenic plants overexpressing the proteinase inhibitor caused a growth stimulation of the larvae (Girard *et al.*, 1998), although the inhibitor used was capable of inhibiting the digestive proteases of the larvae. This indicates that a selection of a proteinase inhibitor purely on in vitro characteristics is very difficult, if not impossible.

Within the group of proteinase inhibitors, plant proteinase inhibitors and within this group, serpins
20 have drawn some interest as possible pest inhibitors. Plant serpins have been studied intensively over the past years. In cereals, serpins are collectively called Z proteins, and constitute 5% of the total albumin in grains (Hejgaard, 1982). Several serpins from wheat, barley, rye, and oat have been identified and characterized (Rasmussen, 1993; Østergaard *et al.*, 2000; Brandt *et al.*, 1990; Lungaard and Svensson, 1989; Rosenkrands *et al.* 1994; Rasmussen *et al.*,
25 1996; Dahl *et al.*, 1996; Hejgaard, 2001; Hejgaard and Hauge, 2002) Biochemical studies with plant serpins from cereals have demonstrated their inhibitory action against different animal proteases such as trypsin, chymotrypsin, cathepsin G, and elastase (Østergaard *et al.*, 2000; Dahl *et al.*, 1996; Hejgaard, 2001; Hejgaard and Hauge, 2002). Also the serpin isolated from squash phloem was reported to be an elastase inhibitor (Yoo *et al.*, 2000)

Whereas in animals, serpins are known to be involved in fundamental biological processes
30 (Patston, 2000; van Gent *et al.*, 2003) no precise role has yet been assigned to plant serpins. The high abundance of serpins in cereal seeds has led to the hypothesis that they could be involved in defense of storage tissue against insect feeding (Østergaard *et al.*, 2000; Hejgaard and Hauge, 2002). Although direct feeding of aphids with CmPS-1 did not affect their survival,
35 the negative correlation between the survival of aphids feeding on squash leaves and expression levels of the phloem serpin CmPS-1 supports the defense theory (Yoo *et al.*, 2000) Gene expression studies in barley have revealed that serpin transcripts are present in developing

grain, but also in vegetative tissues, such as roots, shoots, and leaves. Serpin protein could be detected in phloem cells, meristem, and root cap cells of the young root, and in root cap, coleorhiza, and apical meristem of embryonic roots. Also in young leaves some phloem cells produced serpin proteins (Roberts *et al.*, 2003). Although these expression patterns are
5 intriguing, complete insight into the precise functional role of plant serpins is still lacking.

Based on this possible protection, several serpins have been tested as insect inhibitors, but none of those have been extremely effective. WO9413810 discloses the use of a *Nicotiana glauca* type II serine proteinase inhibitor precursor with at least four domains for increasing plant resistance to pest, but the effect is limited to a retardation in growth of the nymphs tested. US2003/0018990
10 discloses the use of a serine proteinase of *Brassica oleracea* for obtaining resistance to herbivorous insects. Depending upon the parameters measured, and the insects used, the results obtained are comparable or less efficient than the results obtained with Bt. Yoo *et al.* (2000) described the use of *Cucurbita maxima* phloem serpin-1 for inhibiting the piercing-sucking aphid *Myzus persicae*. Although they claim to see a decrease in survival on the transgenic plants
15 in function of the time, they could not demonstrate any toxicity when using the phloem and the effects of survival on the transgenic plants might be due to other factors.

The reactive centre loop (RCL) of serpins is essential for their specificity. The reactive centre loop, as defined here, is defined by the P1 position after which the target proteinase is cleaving the proteinase inhibitor. The RCL as defined here encompasses at least 4 amino acids,
20 preferably 5 amino acids, even more preferably 6 amino acids, including the P1 and aminoterminal of the cleavage site, i.e. P1 is the carboxyterminal amino acid after cleavage. A mutation as used here, is any change in amino acid in this RCL. Preferably, it is a non-conservative change of one or more amine acids. Mutations in the RCL are supposed to block the activity of the serpin, as the RCL acts as substrate for the protease and exerts its inhibiting
25 activity by competition with the natural substrate. Indeed, Vercaemmen *et al.* (2006) demonstrated that mutation of the RCL loop into an IKLA or a VRPR sequence resulted in a significant loss of inhibitory activity.

Surprisingly we found that those mutant serpins do have an inhibiting activity against insect proteases, and that this activity may be far more pronounced than the activity of the wild type
30 serpin. Mutations of the RCL, without – or with limited - changes in the backbone of the serpin allows to design insect specific serpins, and opens the possibility to design plants with a specific resistance against some insects, without affecting non target organisms.

A first aspect of the invention is the use of a recombinant serpin, comprising a mutant reactive center loop, for limiting insect and/or mite provoked damage in plants. A mutant RCL as used
35 here means that the RCL differs from the sequence that occurs in the corresponding wild type serpin. Preferably, said serpin belongs to the inhibitor family IA, as determined by MEROPS (<http://merops.sanger.ac.uk>), showing sequence homology to human alpha1-antitrypsin.

Preferably said serpin is selected of the group consisting of *Arabidopsis thaliana* serpin At1g47710, Barley serpin BZx, Barley serpin BZ7, Wheat serpin WZ2b, *Cucumis sativus* serpin gi|58416137, *Oryza sativa* serpin gi|37700305, *Citrus x paradise* serpin gi|26224736, *Solanum tuberosum* serpin gi|62950609, *Lycopersicon esculentum* serpin gi|14685955, 5 *Nicotiana tabacum* serpin gi|52837819, *Brassica rapa* serpin gi|54416864, *Vitis vinifera* serpin gi|33401535, *Antirrhinus majus* serpin gi|51113970, *Triphysaria versicolor* serpin gi|68033793, *Helianthus paradoxus* serpin gi|33123072, *Gossypium arboretum* serpin gi|13245345, *Populus nigra* serpin gi|60218046 and *Brassica napus* serpin (SEQ ID N° 2). Even more preferably, said the backbone of said serpin is derived from SEQ ID N° 1 (At1g47710).

10 Preferably, said mutant serpin comprises a RCL comprising a sequence selected from the group consisting of IKLA, VRPR, IRPR, WRHR and IKLK. Even more preferably, said mutant serpin comprises a RCL comprising IKLA. Preferably, said insect and/or mite caused damage is caused by a chewing insect. More preferably, said insect or mite belongs to an order selected from the group consisting of the orders orders *Lepidoptera*, *Coleoptera*, *Homoptera* 15 and *Acari*. Even more preferably, said insect or mite is selected from the group consisting of *Spodoptera* spp, *Sesamia* spp., *Ostrinia* spp., *Leptinotarsa* spp., *Tribolium* spp., *Acyrtosiphon* spp., *Bemisia* spp. and *Tetranychus* spp. Most preferably said insects or mites belong to the species *Spodoptera littoralis*, *Sesamia nonagroides*, *Ostrinia nubilalis*, *Leptinotarsa decemlineata*, *Tribolium castaneum*, *Acyrtosiphon pisum*, *Bemisia tabaci* and/or *Tetranychus* 20 *urticae*. Insect damage, as used here, can be any damage, such as visual damage to leaf, stem, seeds or fruit, or a decrease in yield, when compared with the plant that is not affected by said insects or mites. Preferably, said damage is a decrease in yield. Limiting insect damage means that the damage in the treated plant is less severe than in the non-treated control.

25 A preferred embodiment is the use of said serpin, whereby said serpin is applied by spraying. Another preferred embodiment is the use of said serpin, whereby said serpin is overexpressed in a plant. In case of overexpressing, the concentration in the plant material is preferably at least 0.65mg/kg wet weight, even more preferably at least 6.5 mg/kg wet weight, most preferably at least 65mg/kg wet weight.

30 Still another aspect of the invention is a method to develop new mutant serpins with a specific inhibition spectrum, comprising the mutation of one or more amino acids of the RCL. A specific inhibition spectrum as used here means the mutant serpin inhibits other proteinases than the wild type serpin, or shows a significantly higher inhibition towards a proteinase that is inhibited by the wild type serpin. Significantly higher as used here means that the inhibition occurs at 35 significantly lower concentration of the serpin, and/or that a significantly higher inhibition percentage is reached. Preferably, a specific inhibition spectrum means that other proteinases are inhibited than by the wild type mutant. Even more preferably, by the change in inhibition

spectrum, proteinases can be inhibited that are not inhibited by the wild type serpin, when both mutant and wild type serpin are used at the same concentration.

Another aspect of the invention is a transgenic plant, expressing a recombinant serpin, comprising according to the invention. Preferably, said plant is used to limit insect damage
5 caused by insects feeding on the plant.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Inhibition of serine-like protease activities from *S. littoralis* extracts by AtSerp1 (WT) and its derived variant IKLA. Data are the mean \pm SE of duplicate measurements from a
10 unique pool of gut extracts.

Figure 2: Inhibition of serine-like protease activities from *S. nonagrioides* extracts by AtSerp1 (WT) and its derived variant IKLA. Data are the mean \pm SE of duplicate measurements from a unique pool of gut extracts.

Figure 3: Inhibition of cysteine-like protease activities from *L. decemlineata* extracts by
15 AtSerp1 (WT) and its derived variant IKLA. Data are the mean \pm SE of duplicate measurements from a unique pool of gut extracts.

Figure 4: Inhibition of cysteine-like protease activities from *T. castaneum* extracts by AtSerp1 (WT) and its derived variant IKLA. Data are the mean \pm SE of duplicate measurements from a unique pool of gut extracts.

Figure 5: Inhibition of cysteine-like protease activities from *A. pisum* extracts by AtSerp1
20 (WT) and its derived variant IKLA. Data are the mean \pm SE of duplicate measurements from a unique pool of gut extracts.

Figure 6: Inhibition of cysteine-like cathepsin-L protease activities from *B. tabaci* extracts by AtSerp1 (WT) and its derived variant IKLA. Data are the mean \pm SE of duplicate
25 measurements from a unique pool of gut extracts.

Figure 7: Inhibition of cysteine-like protease activities from *T. urticae* extracts by AtSerp1 (WT) and its derived variant IKLA. Data are the mean \pm SE of duplicate measurements from a unique pool of gut extracts.

Figure 8: (A) Micrograph showing a first-instar nymph of *A. pisum* treated for 2 days with
30 10000 μ g/ml serpin mutant IKLA. In intoxicated aphid nymphs, clear symptoms were observed of inhibited growth as compared to control aphids (control) with a significant reduction in aphid size (the aphid size was reduced about 50%) and then followed by aphid mortality (the dead aphid colors brown). **(B)** Dose-response curve for %mortality of the aphid *Acyrtosiphon pisum* when fed for 3 days on an artificial diet comprising the AtSerp1 mutant IKLA. The estimated
35 median lethal concentration (LC50) was 406 μ g/ml with an 95% confidence interval of 271-607 μ g/ml (Logarithm(LC50)=2.608 \pm 0.0785; R2=0.95).

EXAMPLES

Materials and methods to the examples

5

Recombinant Atserpin1 production; construction and production of the mutants

Recombinant Atserpin1 production and purification was done as described in Vercammen et al., 2006.

The cDNA for the ORF of At1g47710 was obtained by RT-PCR with the following forward and reverse primers, provided with the adequate 5' extensions for Gateway™ cloning (Invitrogen):
10 5'-ATGGACGTGCGTGAATC-3' and 5'-TTAATGCAACGGATCAACAAC-3'. After recombination in pDEST17, the plasmid was introduced into *E. coli* strain BL21(DE3)pLysE and production of the HIS6-tagged protein induced by incubation in 0.2 mM isopropyl-β-D-thiogalactopyranoside for 24 h. The protein was purified by metal ion affinity chromatography (TALON™; BD, Franklin
15 Lakes, NJ). Protein concentration and purity were checked by Bradford analysis (BioRAD) and SDS-polyacrylamide gel electrophoresis (PAGE). Point mutagenesis of AtSerpin1 was done using the "megaprimer" method. The reverse mutagenic primers used were:

IKLA, 5'- CTCCATAAGCAATCCTGCTAGCTTTATAACTCC-3';

IKLK, 5'- CTCCATAAGCAATCCTTTTAGCTTTATAACTCC-3'; and

20 VRPR, 5'- CATAAGCAATCCTCTTGGCCTTACAACCTCCAGCTGATGC-3'.

Bacterial production and purification were performed as described for wild-type AtSerpin1.

Insects

A selection of economically important pest insects was made to support the insecticide
25 potency and wide target pest range of AtSerpins. The selected Lepidoptera (caterpillars) and Coleoptera (beetles) are representative pest insects with biting-chewing mouth paths and important in agriculture, horticulture, forestry and stored products. The selected Homoptera (aphids, whiteflies) and Acari (mites) are good representatives for piercing-sucking pests, being very important in agriculture, horticulture, forestry.

30

Lepidoptera:

A colony of the cotton leafworm *Spodoptera littoralis* (CLW) (Lepidoptera: Noctuidae) was reared on a semi-artificial diet at standard conditions in the laboratory. Larvae were collected and frozen at -20°C until analysis.

35 For the artificial feeding tests, newly moulted (0-6h) 3rd instar larvae of the cotton leafworm *Spodoptera littoralis* (Lepidoptera: Noctuidae) (CLW) were selected from a continuous stock colony in the Laboratory of Agrozoology at Ghent University, Belgium, that was kept at

standard conditions of 23±2°C; 65±5% relative humidity and a 16:8 (light:dark) regime (Smagghe et al., 2002).

The cotton leafworm is a polyphagous noctuid species of world economic importance in agriculture and horticulture. Such caterpillars cause high levels of damage in at least 87 crop species belonging to 40 families distributed all over the world. Also many populations developed high levels of resistance towards most insecticide groups (Alford, 2000).

Larvae of Mediterranean corn borer, *Sesamia nonagroides* (MCB) (Lepidoptera: Noctuidae), were frozen and stored at -20 °C until needed.

Larvae of European corn borer, *Ostrinia nubilalis* (Lepidoptera: Crambidae) (ECB), were collected and frozen at -20°C until analysis.

Coleoptera:

Colorado potato beetles *Leptinotarsa decemlineata* (CPB) (Coleoptera: Chrysomelidae) were reared on freshly-cut potato foliage, *Solanum tuberosum*, collected and frozen at -20°C until analysis.

Adults of the red flour beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae) were selected from a colony maintained in the laboratory at standard conditions, frozen and stored at -20°C until needed.

Homoptera:

A laboratory colony of the pea aphid *Acyrtosiphon pisum* (PA) (Homoptera: Aphididae) was reared on pea plants, *Vicia faba*. Adults were selected, frozen and stored at -20°C until needed. Green peach aphid adults, *Myzus persicae* (Homoptera: Aphididae) were selected, frozen and stored at -20°C until needed.

Adults of the sweetpotato whitefly, *Bemisia tabaci* (Homoptera: Aleyrodidae) were selected, frozen and stored at -20°C until needed.

Acari:

Spider mites, *Tetranychus urticae* (Acari: Tetranychidae) were selected from a colony maintained in the laboratory at standard conditions, frozen and stored at -20°C until needed.

Inhibitory activity of Arabidopsis thaliana serpins

Complete guts from CLW, MCB, ECB and CPB larvae were dissected and subsequently homogenized in 0.15 M NaCl, centrifuged at 10,000g for 5 min, and the supernatants pooled and stored frozen (-20°C). For PA, whole insect bodies were used.

Inhibitory activity of wild-type Serpin1 from *Arabidopsis thaliana* and its derived variant IKLA were tested *in vitro* against serine-like protease activities from CLW, MCB (trypsin and

chymotrypsin) and ECB (trypsin and elastase), and against cysteine-like protease activities (cathepsin-B and cathepsin-L) from PA and CPB. *A. thaliana* serpins were preincubated at room temperature with the gut extracts for 15 min, prior to addition of substrate. Serpin concentrations varied from 0.02-20 μ M. Standard fluorometric substrates used were Z-Arg-Arg-amc, Z-Phe-Arg-amc, and N-Suc-Leu-Leu-Val-Tyr-4-Methylcoumaryl-7-amide, from Bachem. For measuring elastase activity the colorimetric substrate Sa2PppNa (N-succinyl-(alanine)²-prolinephenylalanin-p-nitroanilide) were used, from Sigma. All assays were carried out in duplicate with pooled gut extracts. Fluorescence was monitored for 30 min at λ_{ex} of 380 nm and λ_{em} of 460 nm in a microtiterplate reader. For measuring the elastase activity in ECB, the colorimetric substrate Sa2PppNa (N-succinyl-(alanine)²-proline-phenylalanin-p-nitroanilide) were used, from Sigma.

Absorbance was measured at 410 nm for elastase activity in a microplate reader. Insect protease activities were determined at 30°C, at pH 10.5 for CLW, MCB and ECB, and at pH 7.5 for PA and CPB, in 100 μ l of reaction mixture.

Inhibitory activity of the derived variants IKLK and VRPR were also tested in vitro against trypsin activity of CLW and cathepsin B activity of PA. Standard colorimetric substrates used were BApNa (*N* α -benzoyl-DL-arginine-*p*-nitroanilide) and ZAA2MNA (*N*-carbobenzoxyalanine-alanine-arginine 4-methoxy- β -naphthyl amide), from Sigma. All assays were carried out in triplicate with pooled gut extracts. Serpin concentrations were 5 and 10 μ M. Absorbance was measured at 410 nm for trypsin activity and 520 nm for cathepsin B activity in a microplate reader. Insect protease activities were determined at 30 °C, at pH 10.5 for CLW and at pH 7.5 for PA, in 100 μ l of reaction mixture.

Insect bioassay with pea aphid in a feeding apparatus with artificial assay supplemented with recombinant serpin mutant IKLA protein.

A colony of the pea aphid (*Acyrtosiphon pisum*; Homoptera: Aphididae) was maintained continuously at the Laboratory of Agrozoology (Ghent University) on young *Vicia faba* L. plants that are grown in a plant growth room at 23-25°C, 60-65% relative humidity, and a photoperiod of 16h light:8 h dark. Mature aphids are put on plants for 24 h and the resulting offspring nymphs (aged 0-24 h) are used in the insect bioassay experiments.

As food for the aphids, a standard diet previously developed for *A. pisum* (Febvay *et al.*, 1988) was used as the basal diet to which the recombinant serpin IKLA mutant protein was added. Then the prepared liquid artificial diet was filter-sterilized through a 0.2- μ m filter (Millipore Corp., Bedford, MA, USA). Aliquots of 1.5 ml can be stored in the freezer at -20°C for a period up to 6 months. After defrosting at room temperature, the artificial diet is ready to be used.

The feeding apparatus was prepared in-house using plexiglass cylinders (3 cm high and 3 cm diameter). The food sachet was made under sterile conditions and consists of two layers of

parafilm membrane on top of the container. 200 µl of the artificial diet was sandwiched between the two layers.

To challenge aphids to recombinant serpin protein, a series of concentrations (100-10000 µg/ml) of the protein (made in PBS buffer) was prepared in the artificial diet. In the treatments, 200 µl of each concentration was used to make a food sachet. In the controls, the diet was supplemented with a corresponding amount of buffer. Per sucking apparatus, 15 synchronized neonate nymphs (aged 0-24 h) were transferred onto the artificial diet. For each concentration, three replicates were carried out, and the experiment was performed two times independently from each other; a total of 90 aphids was tested per concentration. Aphids were checked daily during 3 consecutive days for dead individuals to calculate mortality percentages.

Differences between treatments were considered significant at $p=0.05$. The median lethal concentration 50% (LC_{50}) and the 95% confidence interval were determined from the dose-response curve using the nonlinear regression analysis in Prism v4 (GraphPad, San Diego, CA, USA) (Smaghe and Degheele, 1994).

Example 1: inhibition of enzymes of the cotton leafworm, *Spodoptera littoralis* (Lepidoptera: Noctuidae)

As shown in Figure 1, AtSerp1 exerts a clear inhibition of serine-like trypsin activities already at low concentration with 50% inhibition at about 1 µM concentrations. Maximal inhibition of trypsin activity was scored with ≥ 10 µM. In contrast the IKLA mutant did not show potency to inhibit the trypsin protease activities of *S. littoralis*. Here fluorometric substrate was employed to measure trypsin activities.

For chymotrypsin activity inhibition the concentration of AtSerp1 needed to inhibit 50% was estimated at 10-20 µM (Figure 1). For the IKLA derived variant of AtSerp1, its potency to inhibit trypsin activity was lower than the original AtSerp1, but its activity against chymotrypsin was similar.

With the use of colorimetric substrates, table 1 confirms the high potency of AtSerp1 to inhibit trypsin protease activities from *S. littoralis* extracts with 52% inhibition at 1.25 µM and 83% at 5 µM. With the three different derived variants (mutant IKLA, IKLK and VRPR) the activity was lower compared to AtSerp1, and for the mutants WRHR and IRPR there was no activity present. The tests for inhibition of chymotrypsin activities with AtSerp1 and the IKLA mutant showed activity with 40- 56% inhibition with the highest concentration tested (10 µM).

Table 1. Inhibition of serine-like protease activities from extracts of larvae of the cotton leafworm, *Spodoptera littoralis* (Lepidoptera), by AtSerp1 and derived mutants.

	% Inhibition					
	Trypsin			Chymotrypsin		
	1.25 μ M	5 μ M	10 μ M	1.25 μ M	5 μ M	10 μ M
AtSerp1	52 \pm 1	83 \pm 1	90 \pm 1	10 \pm 21	31 \pm 20	40 \pm 15
Mutant IKLA	18 \pm 1	32 \pm 2	40 \pm 2	11 \pm 16	41 \pm 5	56 \pm 5
Mutant IKLK	--	64 \pm 2	69 \pm 3	--	--	--
Mutant VRPR	ni	33 \pm 1	34 \pm 3	ni	--	--
Mutant WRHR	ni	--	--	ni	--	--
Mutant IRPR	ni	--	--	ni	--	--

-- = not determined

ni = no inhibition

5

Example 2: inhibition of the enzymes of the Mediterranean corn borer, *Sesamia nonagrioides* (Lepidoptera: Noctuidae)

10 Figure 2 demonstrates a very strong inhibition by AtSerp1 of trypsin activities already at low concentration with 50% inhibition at about 0.1 μ M concentrations. Maximal inhibition of trypsin activity was scored already with 0.5-1 μ M. The IKLA derived variant scored low inhibitory activity.

For chymotrypsin activity inhibition the concentration of AtSerp1 needed to inhibit \geq 50% was estimated at \geq 10 μ M. Interestingly, for the IKLA derived variant of AtSerp1, its potency to inhibit chymotrypsin activity was higher than the original AtSerp1.

Table 2 clearly provides confirmation of the high potency of AtSerp1 to inhibit trypsin protease activities from *S. nonagrioides* extracts. With the derived IKLA variant the activity was lost. The tests for inhibition of chymotrypsin activities with AtSerp1 showed no activity; however with the mutant IKLA a high activity with 70% inhibition was scored at 10 μ M.

20

Table 2. Inhibition of serine-like protease activities from extracts of larvae of the Mediterranean corn borer, *Sesamia nonagrioides* (Lepidoptera), by AtSerp1 and derived mutants.

	% Inhibition					
	Trypsin			Chymotrypsin		
	1.25 μ M	5 μ M	10 μ M	1.25 μ M	5 μ M	10 μ M
AtSerp1	90 \pm 1	97 \pm 1	98 \pm 1	ni	ni	ni
Mutant IKLA	ni	ni	ni	ni	32 \pm 10	70 \pm 20
Mutant WRHR	ni	--	--	ni	--	--
Mutant IRPR	38 \pm 6	--	--	ni	--	--
Mutant VRPR	ni	--	--	ni	--	--

ni = no inhibition

-- = not determined

25

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Example 3: Inhibition of the enzymes of the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Crambidae)

With use of colorimetric substrates for trypsin and chymotrypsin activities, AtSerp1 shows limited potency to inhibit trypsin protease activities from *O. nubilalis* extracts with 28% at 2 μ M (Table 3). With the derived IKLA variant the activity was much higher reaching 51% inhibition at 2 μ M. The tests for inhibition of chymotrypsin activities with AtSerp1 showed no activity, whereas the mutant IKLA exerted 21% inhibition at 2 μ M.

Table 3. Inhibition of serine-like protease activities from *O. nubilalis* extracts by AtSerp1 and its derived variant IKLA.

	% Inhibition	
	Trypsin 2 μ M	Elastase 2 μ M
AtSerp1	28 \pm 2	ni
Mutant IKLA	51 \pm 1	21 \pm 1

ni= no inhibition

Example 4: Inhibition of the enzymes of the Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae)

As shown in Figure 3 with use of fluorometric substrates, AtSerp1 provokes 50% inhibition of cysteine-like cathepsin-L protease activities at ≥ 10 μ M in the Colorado potato beetle gut extracts.

For the cathepsin-B activity inhibition, the concentration of AtSerp1 needed to inhibit 50% was estimated >10 μ M. For the IKLA derived variant of AtSerp1, its potency to inhibit both cysteine-like protease activities, cathepsin-B and cathepsin-L activity, was higher than the original AtSerp1 (Figure 3).

With use of colorimetric substrates, AtSerp1 inhibits cathepsin-B protease activities with 40% already at relatively low concentrations of 1.25 μ M (Table 4). For cathepsin-L activities the potency of AtSerp1 for inhibition was lower, yielding 4% at 1.25 and increasing up to 48% at 10 μ M. Interestingly with the derived variant IKLA the activity was higher as with the original AtSerp1 (Table 4). With the lowest concentration tested (1.25 μ M) already 62% inhibition of cathepsin-B was scored. The tests for inhibition of cathepsin-L activities with IKLA scored 66% at 10 μ M.

Table 4. Inhibition of cysteine-like protease activities from extracts of the Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera), by the AtSerp1 and derived mutant IKLA.

	% Inhibition					
	Cathepsin-B			Cathepsin-L		
	1.25 μ M	5 μ M	10 μ M	1.25 μ M	5 μ M	10 μ M
AtSerp1	40 \pm 8	47 \pm 8	46 \pm 11	4 \pm 10	34 \pm 4	48 \pm 4
Mutant IKLA	62 \pm 5	77 \pm 3	70 \pm 2	18 \pm 2	44 \pm 2	66 \pm 1

Table 5. Inhibition of cysteine-like protease activities (cathepsin-L) from extracts of adults of the red flour beetle, *Tribolium castaneum* (Coleoptera), by AtSerpin1 and derived mutants.

	% Inhibition										
	Cathepsin-L										
	0.04 μM	0.08 μM	0.16 μM	0.31 μM	0.63 μM	1.25 μM	2.5 μM	5 μM	10 μM	20 μM	
AtSerpin1	ni	ni	ni	ni	ni	ni	16 ± 1	68 ± 1	73 ± 8	76 ± 12	
Mutant KLA	ni	12 ± 1	32 ± 4	57 ± 5	63 ± 2	68 ± 9	68 ± 7	72 ± 17	70 ± 8	76 ± 15	
Mutant WRHR	--	--	--	--	--	ni	--	--	--	--	
Mutant IRPR	--	--	--	--	--	73 ± 1	--	--	--	--	
Mutant VRPR	--	--	--	--	--	38 ± 2	--	--	--	--	

ni = no inhibition

-- = not determined

Example 5: Inhibition of the enzymes of the red flour beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae)

5 Figure 4 shows that the cysteine-like cathepsin-L activity is inhibited by the AtSerp1 with an IC₅₀=4 μM. Very interestingly, the IKLA mutant is >10 times more active and the estimated IC₅₀ yielded 0.2-0.3 μM to inhibit 50% of cathepsin-L activity. For the cathepsin-B activities, no inhibitory activity was scored with IKLA derived mutant of AtSerp1 when tested up to 20 μM. The results with the other mutant serpins are summarized in Table 5.

10

Example 6: inhibition of the enzymes of the pea aphid, *Acyrtosiphon pisum* (Homoptera: Aphididae)

As shown in Figure 5, AtSerp1 exerts inhibition of cysteine-like cathepsin-L protease activities. However, it was strange in this experiment that the effect with AtSerp1 flattened around 40% inhibition. Otherwise it was clear that the inhibitory activity of AtSerp1 was stronger than for the IKLA mutant.

15

For the cysteine-like cathepsin-B activity inhibition of aphid *A. pisum* extracts, the concentration of AtSerp1 needed to inhibit 50% was estimated at around 1 μM (Figure 5). For the IKLA derived variant of AtSerp1, its potency to inhibit cathepsin-B activity was lower than the original AtSerp1.

20

With use of colorimetric substrates, table 6 confirms the high potency of AtSerp1 to inhibit cathepsin-B protease activities from *A. pisum* aphid extracts. 50% inhibition of cathepsin-B was scored with relatively low concentrations of 1.25 μM. For cathepsin-L activities the % of inhibition was somewhat lower yielding 39-42% at 1.25-10 μM.

25

As shown in table 6, with the derived variant IKLK the activity was similar as with the original AtSerp1, whereas with IKLA and VRPR the activities were lower. The tests for inhibition of cathepsin-L activities with IKLA scored somewhat similar as AtSerp1.

Table 6. Inhibition of cysteine-like protease activities from extracts of adults of the pea aphid, *Acyrtosiphon pisum* (Homoptera), by AtSerp1 and derived mutants.

30

	% Inhibition					
	Cathepsin-B			Cathepsin-L		
	1.25 μM	5 μM	10 μM	1.25 μM	5 μM	10 μM
AtSerp1	50 ± 1	55 ± 5	55 ± 2	42 ± 3	42 ± 2	39 ± 4
Mutant IKLA	24 ± 6	31 ± 1	36 ± 3	24 ± 6	34 ± 4	45 ± 1
Mutant IKLK	--	49 ± 4	66 ± 4	--	--	--
Mutant VRPR	27 ± 1	14 ± 2	23 ± 1	16 ± 1	--	--
Mutant WRHR	26 ± 2	--	--	ni	--	--
Mutant IRPR	32 ± 2	--	--	31 ± 2	--	--

ni = no inhibition

-- = not determined

Example 7: Inhibition of the enzymes of the sweetpotato whitefly, *Bemisia tabaci* (Homoptera: Aleyrodidae)

5 Figure 6 demonstrates that the cysteine-like cathepsin-L activity is inhibited by the AtSerp1 with the highest concentration tested (20 μ M) 45% inhibition was scored. Very interestingly, the IKLA mutant is about 5-10 times more active and the estimated IC50 is around 5 μ M to inhibit 50% of cathepsin-L activity. The results are further summarized in Table 7.

10 **Example 8: spider mites, *Tetranychus urticae* (Acari: Tetranychidae)**

In spider mites, AtSerp1 is active to inhibit cathepsin-L protease activities with 50% inhibition at about 2 μ M (Figure 7). Very interesting, the IKLA derived mutant of AtSerp1 is even more active with an IC50 <1 μ M; which is 2-3 times higher in activity. For the cathepsin-B activities, no inhibitory activity was scored with IKLA derived mutant of AtSerp1 when tested up to 20
15 μ M and also with WRHR, VRPR, IRPR derived mutant of AtSerp1 when tested at 1 μ M. The results for the IKLA mutant, in comparison with AtSerp1 and other mutants (at a concentration of 1.25 μ M) are summarized in Table 8.

20 **Example 9: Inhibition of growth and development, and mortality in pea aphid *Acyrtosiphon pisum* fed on a diet comprising AtSerp1 mutant IKLA.**

Inhibition of aphid nymphal growth and development was measured on a diet comprising recombinant AtSerp1 mutant IKLA as compared to an artificial control diet where the nymphal growth/development was normal. The inhibitory effects were already visible with concentrations of 500 μ g/ml. In these affected nymphs a conspicuous inhibition of size
25 increase can be seen already after 2-3 day of treatment. As exemplified in Figure 8A, the intoxicated aphids show an average reduction of 50% and then this was followed by death of these individuals; the dead aphids color brown. The median lethal concentration (LC50) of IKLA protein in diet was estimated to be 406 μ g/ml (95% confidence interval: 271-607 μ g/ml; R2=0.95) (Fig. 8B)

30

Table 7. Inhibition of cysteine-like protease activities (cathepsin-L) from extracts of adults of the sweetpotato whitefly, *Bemisia tabaci* (Homoptera) by AtSerp1 and derived mutant IKLA.

	% Inhibition										
	Cathepsin-L										
	0.04 μ M	0.08 μ M	0.16 μ M	0.31 μ M	0.63 μ M	1.25 μ M	2.5 μ M	5 μ M	10 μ M	20 μ M	
AtSerp1	ni	ni	ni	ni	ni	ni	7 \pm 1	20 \pm 1	34 \pm 1	43 \pm 1	
Mutant IKLA	ni	ni	3 \pm 1	10 \pm 1	15 \pm 0	27 \pm 1	38 \pm 2	48 \pm 2	60 \pm 7	70 \pm 5	

ni = no inhibition

-- = not determined

Table 8. Inhibition of cysteine-like protease activities (cathepsin-L) from extracts of adults of the spider mites, *Tetranychus urticae* (Acari) by AtSerp1 and derived mutants.

	% Inhibition													
	Cathepsin-L													
	0.02 μ M	0.04 μ M	0.08 μ M	0.16 μ M	0.31 μ M	0.63 μ M	1.25 μ M	2.5 μ M	5 μ M	10 μ M	20 μ M			
AtSerp1	ni	ni	ni	10 \pm 1	13 \pm 1	17 \pm 1	32 \pm 1	48 \pm 1	58 \pm 1	67 \pm 3	73 \pm 3			
Mutant IKLA	9 \pm 1	11 \pm 1	12 \pm 1	22 \pm 2	33 \pm 2	44 \pm 3	51 \pm 4	59 \pm 5	71 \pm 10	75 \pm 6	79 \pm 26			
Mutant VRPR	--	--	--	--	--	--	22 \pm 3	--	--	--	--			
Mutant WRHR	--	--	--	--	--	--	8 \pm 1	--	--	--	--			
Mutant IRPR	--	--	--	--	--	--	11 \pm 1	--	--	--	--			

ni = no inhibition

-- = not determined

REFERENCES

- Alford D.V. (2000). Pest and disease management handbook. British Crop Protection Council. Blackwell Science. Oxford. 615 pp.
- Brandt, A., Svendsen, I. and Hejgaard, J. (1990). A plant serpin gene. Structure, organisation and expression of the gene encoding barley protein Z. Eur. J. Biochem. 194, 499-505.
- Dahl, S., Rasmussen, S.W. and Hejgaard, J. (1996). Heterologous expression of three plant serpins with distinct inhibitory specificities. J. Biol. Chem. 271, 25083-25088.
- Dulmage, H.T. (1981) Production of bacteria for biological control of insects. In "Biological control in crop production", Paparrizas, D.C. ed., pp 129-141.
- Febvay, G., Delobel, B., and Rahbé, Y. (1988). Influence of the amino-acid balance on the improvement of an artificial diet for a biotype of *Acyrtosiphon pisum* (Homoptera, Aphididae). Can. J. Zool. 66, 2449-2453.
- Girard, C., Le Metayer, M., Zaccomer, B, Barlet, B., Barlet, E., Williams, I., Bonade-Bottino, M., Pham-Delegue, M.H. and Jouanin, L. (1998). Growth stimulation of beetle larvae reared on a transgenic oilseed rape expressin a cysteine proteinase inhibitor. J. Insect Physiol. 44, 263-270.
- Goldberg, L.J. and Margalit, J. (1977). A bacterial spore demonstrating rapid larvicidal activity against *Anopheles segentii*, *Uranotaenia unguiculata*, *Culex univittatus*, *Aedes aegypti* and *Culex pipiens*. Mosquito News 37, 355-358.
- Hejgaard, J. (1982). Purification and properties of protein Z – a major albumin of barley endosperm. Physiol. Plant 54, 174-182.
- Hejgaard, J. (2001). Inhibitory serpins from rye grain with glutamine as P1 and P2 residues in the reactive center. FEBS Lett. 488, 149-163.
- Hejgaard, J. and Hauge, S. (2002). Serpins of oat (*Avena sativa*) grain with distinct reactive centres and inhibitory activity. Physiol. Plant 116, 155-163.
- Haq, S.K., Atif, S.M. and Khan, R.H. (2004). Protein inhibitor genes in combat against insects, pest, and pathogens: natural and engineered phytoprotection. Arch. Biochem. Biophys. 431, 145-159.
- Krieg, A., Huger, A.M. and Schnetter, W. (1987). *Bacillus thuringiensis* var san diego strain M-7 is identical to the formerly in Germany isolated strain BI 256-82, *B. thuringiensis* subsp. *tenebrionis*, which is pathogenic to coleopteran insects. Z. Angew. Ent. 104, 417-425
- Lungard, R. and Svensson, B. (1989). A 39kD barley seed protein of the serpin superfamily inhibits alpha-chymotrypsin. Carlsberg Res. Commun. 54, 173-180.

- Mathavan, S., Velpandi, A., Johnson, J.C. (1987). Sub toxic effects of *Bacillus spaericus* 1593 M on feeding growth and reproduction of *Laccotrephes griseus* (Hemiptera: Nepidae). *Exp. Biol.* 46, 149-153.
- 5 - Østergaard, H., Rasmussen, S.K. Roberts, T.H. and Hejgaard, J. (2000). Inhibitory serpins from wheat grain with reactive centers resembling glutamine-rich repeats of prolamins storage proteins. Cloning and characterization of five major molecular forms. *J. Biol. Chem.* 275, 33272-33279.
- Patston, P.A. (2000) Serpins and other serine protease inhibitors. *Immunol. Today* 21, 345-354.
- 10 - Rasmussen, S.K. (1993) A gene coding for a new plant serpin. *Biochim. Biophys. Acta* 1172, 151-154.
- Rasmussen, S.K., Dahl, S.W., Nørgard, A. And Hejgaard, J. (1996). A recombinant wheat serpin with inhibitory activity. *Plant Mol. Biol.* 30, 673-677.
- Roberts, T.H., Martilla, S., Rasmussen, S.K. and Hejgaard, J. (2003). Differential gene expression for suicide substrate serine proteinase inhibitors (serpins) in vegetative and grain tissues of barley. *J. Exp. Bot.* 54, 2251-2263.
- 15 - Rosenkrands, I. Heygaard, J., Rasmussen, S.K. and Bjørn, S.E. (1994). Serpins from wheat grain, *FEBS Lett.* 343, 75-80.
- Smaghe, G. and Degheele, D. (1994). Action of a novel nonsteroidal ecdysteroid mimic, tebufenozide (RH-5992), on insects of different orders. *Pestic. Sci.* 42, 85-92.
- 20 - Smaghe, G., Decombel, L., Carton, B., Voigt, B., Adam, G. and Tirry, L. (2002). Action of brassinosteroids in the cotton leafworm *Spodoptera littoralis*. *Insect Biochem. Molec. Biol.* 32, 199-204.
- Van Gent, D., Sharp, P., Morgan, K. and Kalsheker, N. (2003). Serpins: structure, function and molecular evolution. *Int. J. Biochem. Cell. Biol.* 35, 1536-1547.
- 25 - Vercammen, D., Belenghi, B., van de Cotte, B., Beunens, T., Gavigan, J.A., De Rycke, R., Brackenier, A., Inzé, D., Harris, J.L. and Van Breusegem, F. (2006). Serpin 1 of *Arabidopsis thaliana* is a suicide inhibitor for metacaspase 9. *J. Mol. Biol.*
- Yoo, B.C., Aoki, K., Xiang, Y., Campbell, L.R., Hull, R.J., Xoconostle-Cazares, B., Monzer, J., Lee, J.Y., Ullman, D.E. and Lucas, W.J. (2000). Characterization of *Cucurbita maxima* Phloem Serpin-1 (CmPS-1). A developmentally regulated elastase inhibitor. *J. Biol. Chem.* 275, 35122-35128.
- 30

CLAIMS

1. The use of a recombinant serpin, comprising a mutant reactive centre loop for limiting insect and/or mite provoked damage in plants.
2. The use according to claim 1, whereby said mutant serpin is selected of the group consisting of *Arabidopsis thaliana* serpin At1g47710, Barley serpin BSZx, Barley serpin BSZ7, Wheat serpin WSZ2b, *Cucumis sativus* serpin gi|58416137, *Oryza sativa* serpin gi|37700305, *Citrus x paradise* serpin gi|26224736, *Solanum tuberosum* serpin gi|62950609, *Lycopersicon esculentum* serpin gi|14685955, *Nicotiana tabacum* serpin gi|52837819, *Brassica rapa* serpin gi|54416864, *Vitis vinifera* serpin gi|33401535, *Antirrhinus majus* serpin gi|51113970, *Triphysaria versicolor* serpin gi|68033793, *Helianthus paradoxus* serpin gi|33123072, *Gossypium arboreum* serpin gi|13245345, *Populus nigra* serpin gi|60218046 and *Brassica napus* serpin (SEQ ID N° 2).
3. The use according to claim 2, whereby said mutant serpin is derived from SEQ ID N° 1 (At1g47710)
4. The use of a recombinant serpin, comprising a reactive centre loop comprising a sequence selected from the group consisting of IKLA, VRPR, IRPR, WRHR and IKLK for limiting insect and/or mite provoked damage in plants
5. The use according to any of the previous claims, whereby said insect and/or mite provoked damage is caused by a chewing insect.
6. The use according to claim 5, whereby said insect or mite belongs to an order selected from the group consisting of the orders orders Lepidoptera, Coleoptera, Homoptera and Acari.
7. The use according to claim 6, whereby said insect or mite is selected from the group consisting of *Spodoptera* spp, *Sesamia* spp., *Ostrinia* spp., *Leptinotarsa* spp., *Tribolium* spp., *Acyrtosiphon* spp., *Bemisia* spp. and *Tetranychus* spp.
8. The use according to any of the previous claims, whereby said mutant serpin is applied by spraying.
9. The use according to claim 1-6, whereby said serpin is overexpressed in a plant.
10. A method to develop new mutant serpins with a specific inhibition spectrum, comprising the mutation of one or more amino acids of the reactive centre loop.
11. A transgenic plant, expressing a recombinant serpin, comprising a mutant reactive center loop.
12. A transgenic plant, according to claim 11, whereby said recombinant serpin comprises a reactive center loop comprising a sequence selected from the group consisting of IKLA, VRPR, IRPR WRHR and IKLK.

Fig. 1:

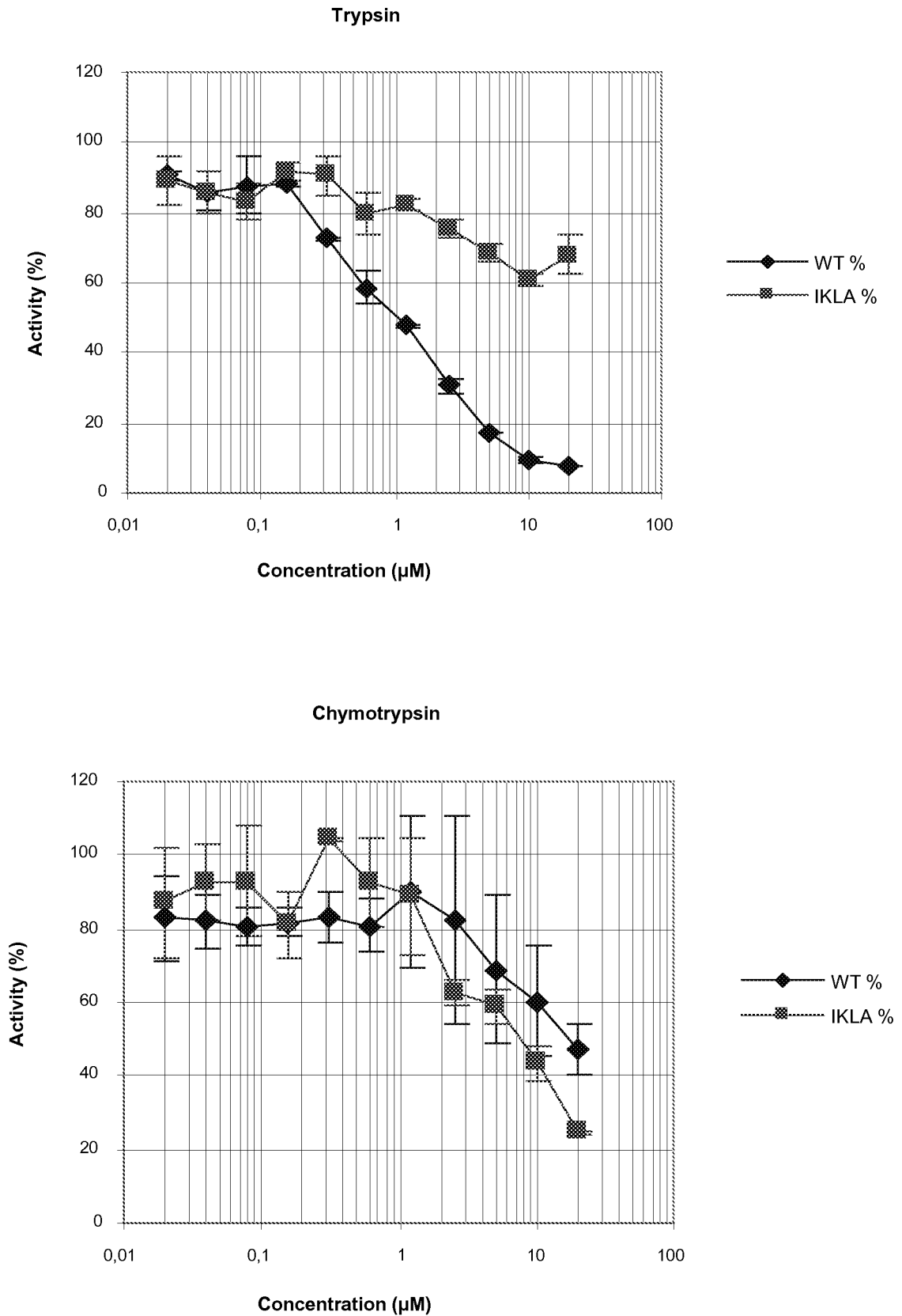


Fig. 2:

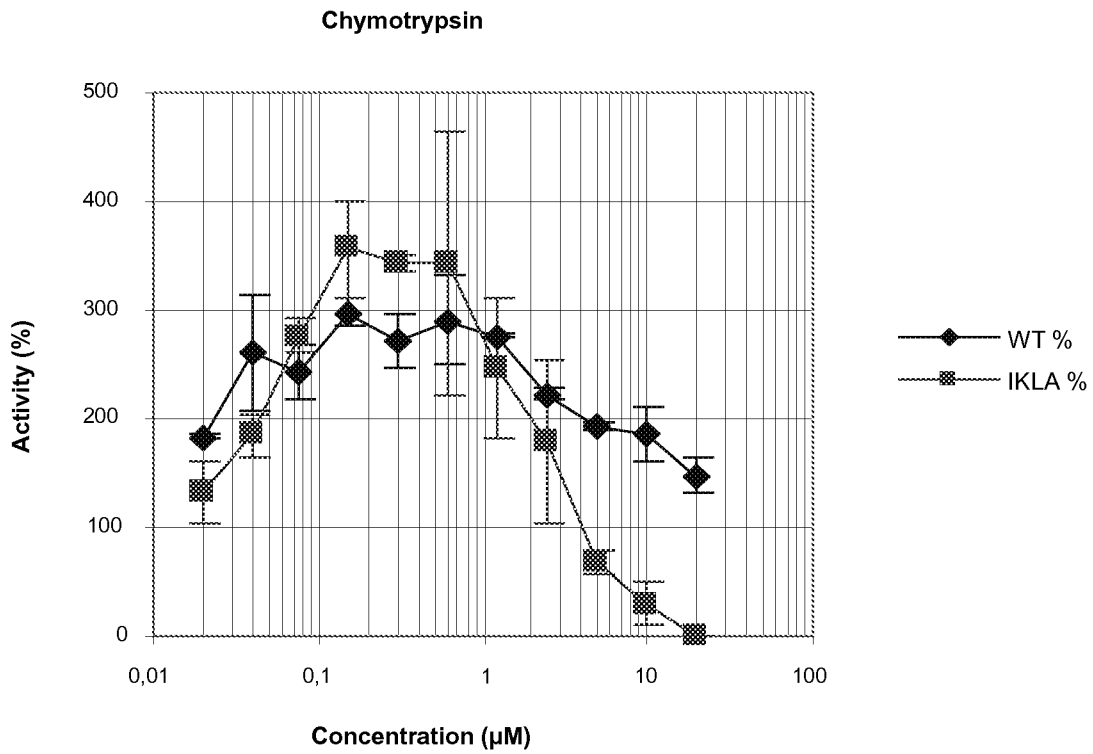
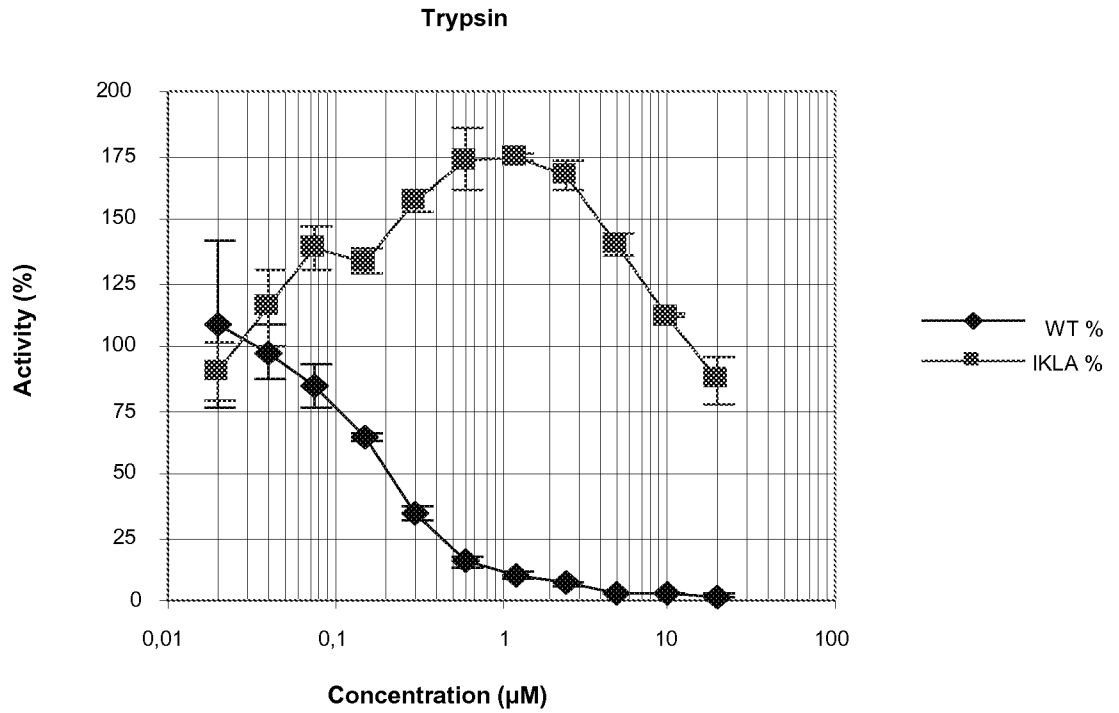


Fig. 3:

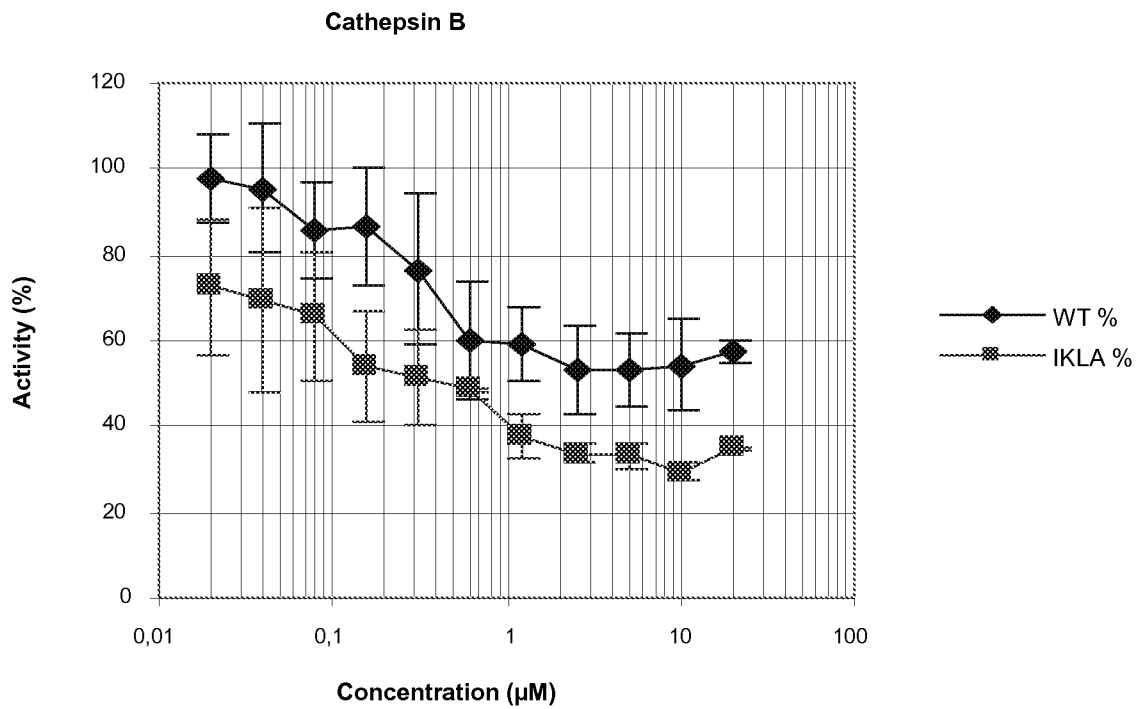
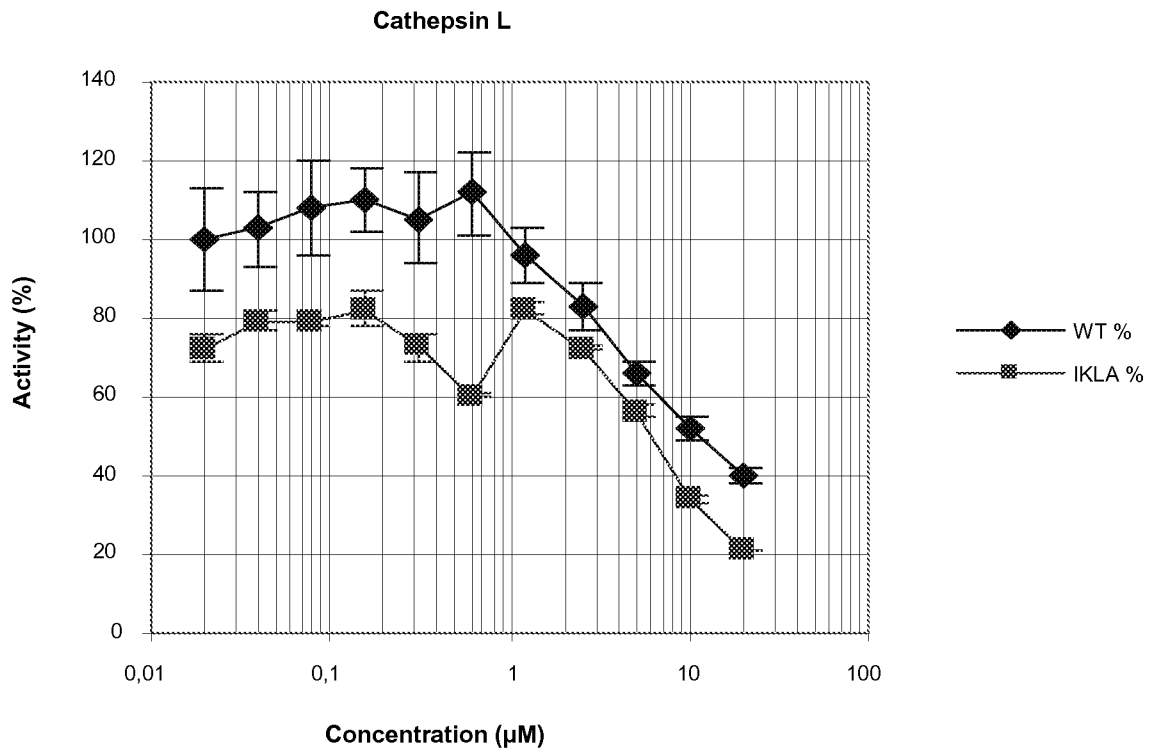


Fig. 4:

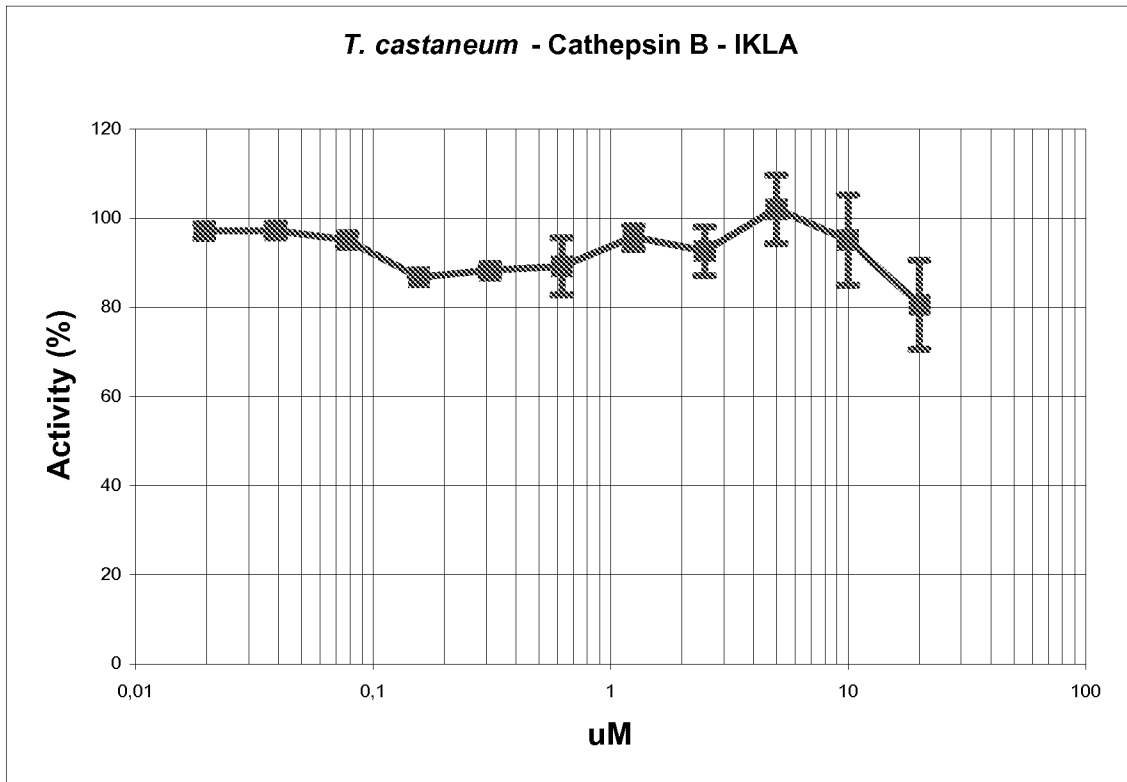
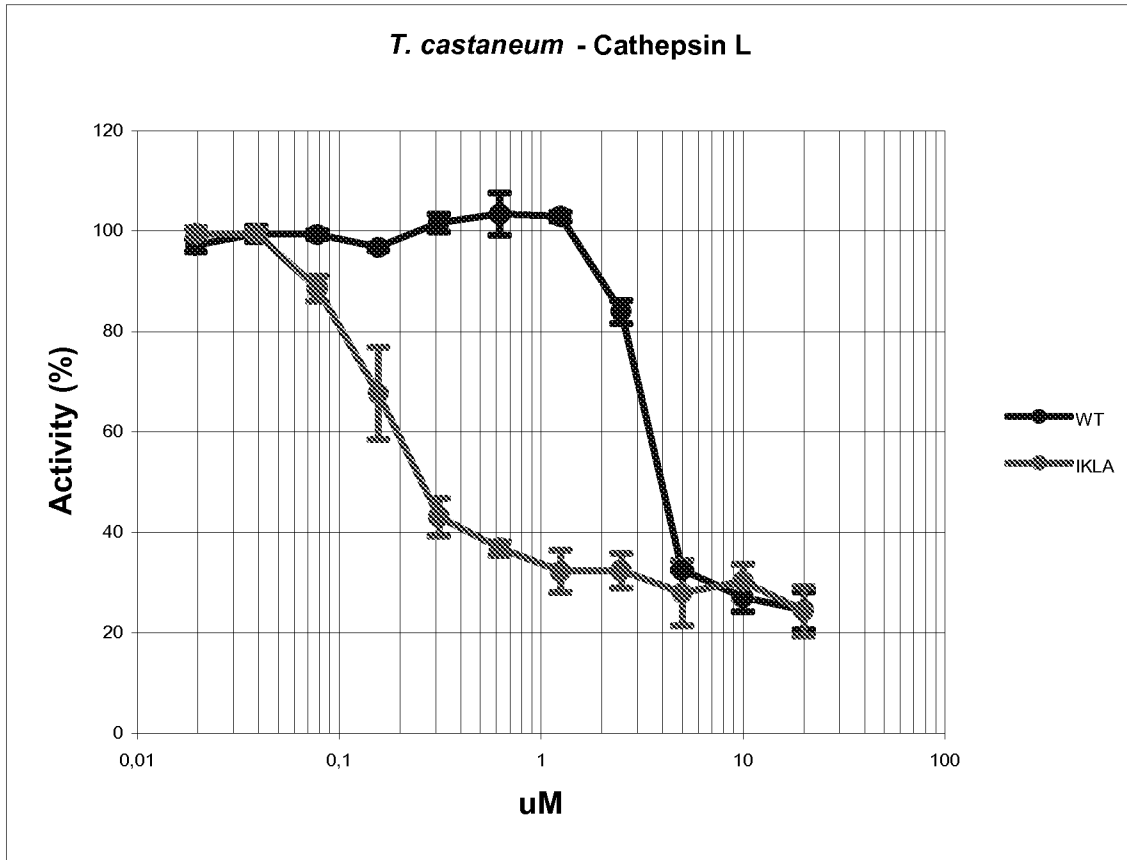


Fig. 5:

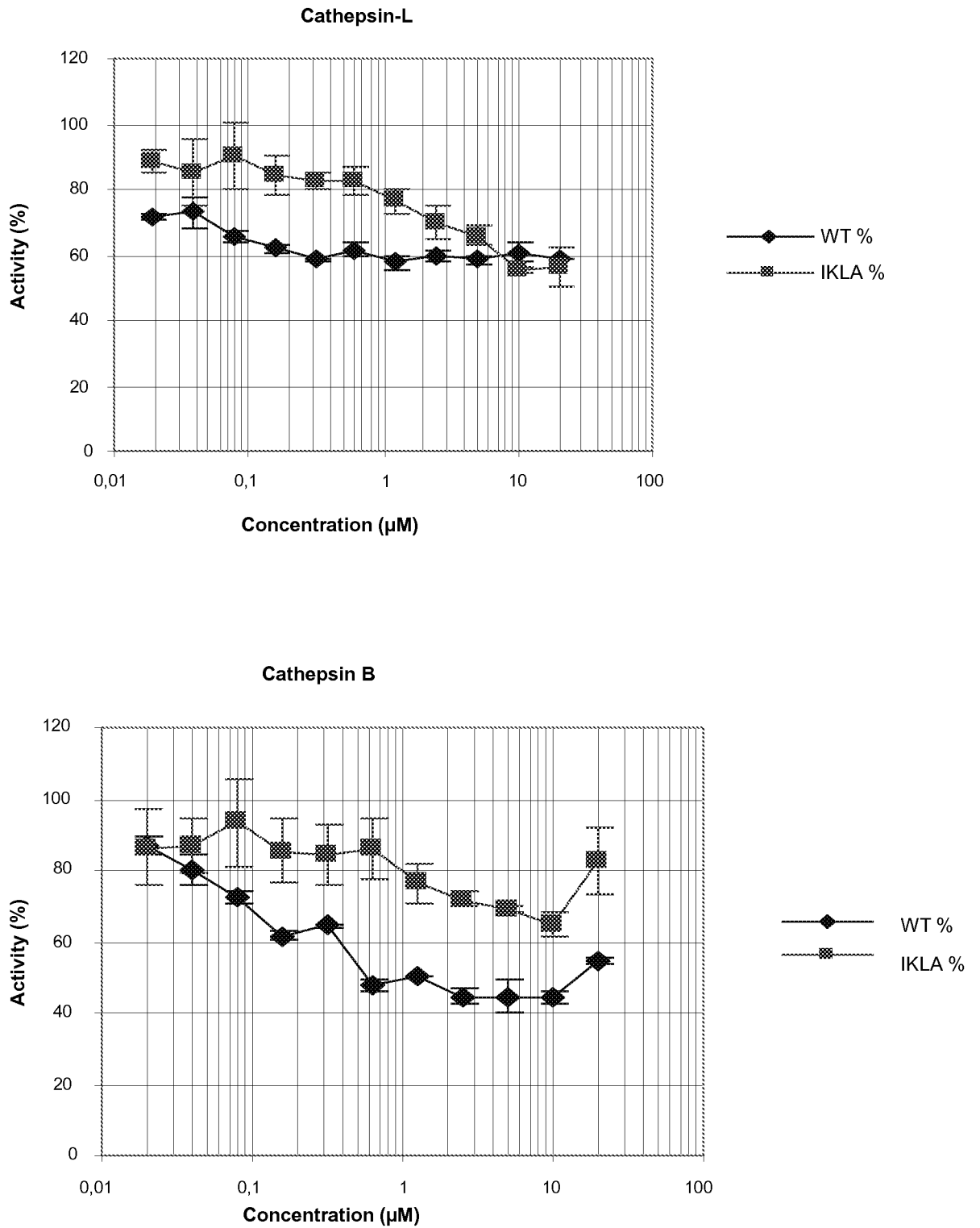


Fig. 6:

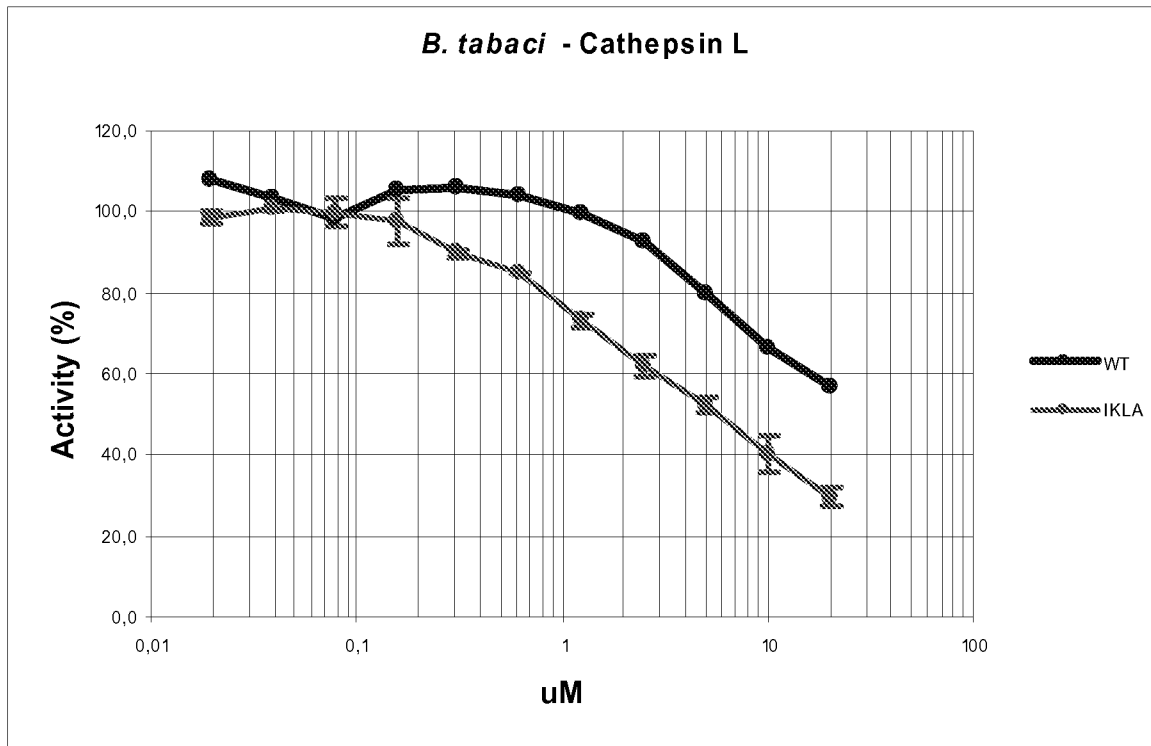


Fig. 7:

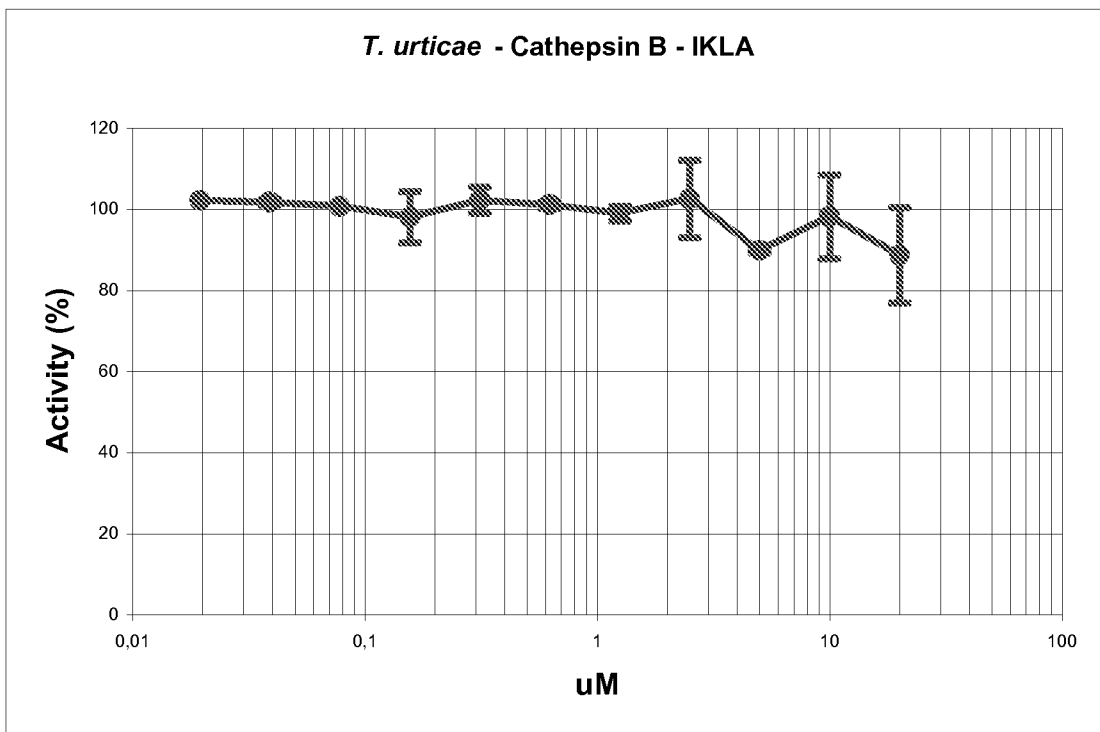
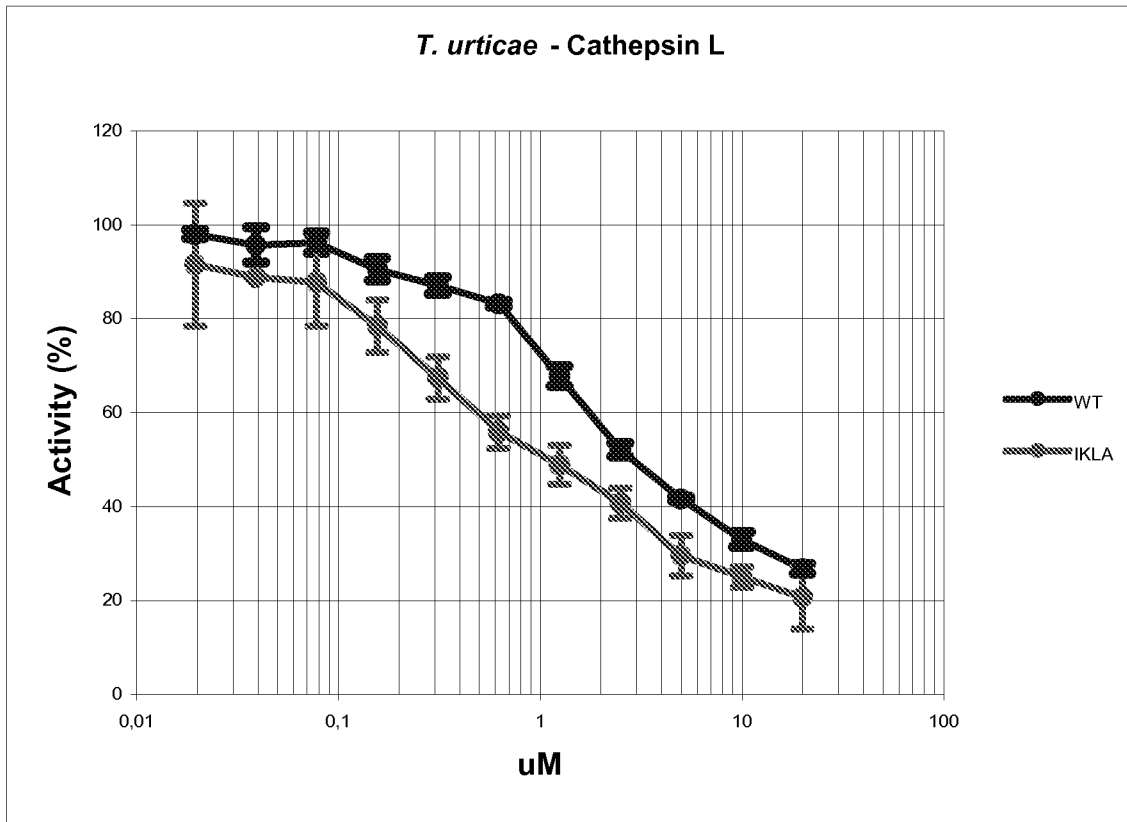
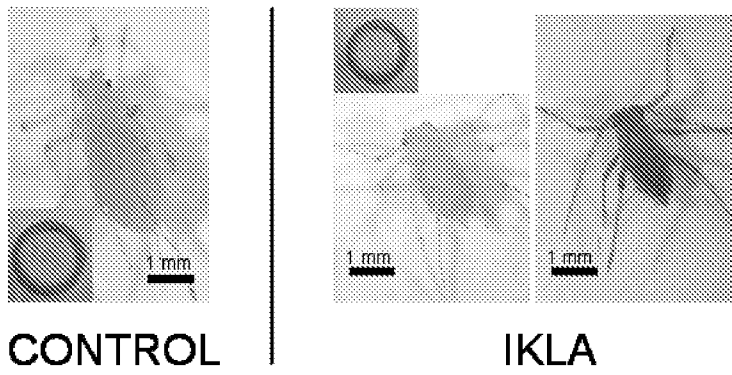
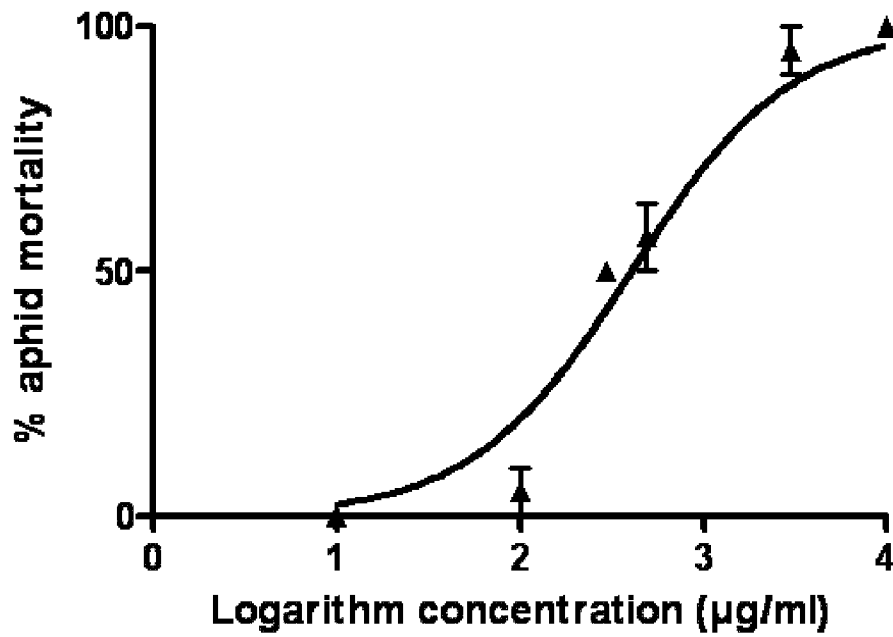


Fig. 8:

A



B



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2008/064728

A. CLASSIFICATION OF SUBJECT MATTER INV. C12N15/82 C07K14/81		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) C12N C07K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, BIOSIS, EMBASE, Sequence Search, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VERCAMMEN DOMINIQUE ET AL: "Serp1n1 of Arabidopsis thaliana is a suicide inhibitor for metacaspase 9" JOURNAL OF MOLECULAR BIOLOGY, LONDON, GB, vol. 364, no. 4, 8 September 2006 (2006-09-08), pages 625-636, XP002425099 ISSN: 0022-2836 cited in the application abstract	10
Y	page 628, right-hand column, line 27 - line 34 page 629, right-hand column, line 30 - line 46 page 632, right-hand column, line 31 - line 46 ----- -/--	1-9, 11, 12
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.		
* Special categories of cited documents : *A* document defining the general state of the art which is not considered to be of particular relevance *E* earlier document but published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. *&* document member of the same patent family		
Date of the actual completion of the international search 3 February 2009		Date of mailing of the international search report 20/02/2009
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Mundel, Christophe

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2008/064728

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>YOO BYUNG-CHUN ET AL: "Characterization of Cucurbita maxima phloem serpin-1 (CmPS-1): A developmentally regulated elastase inhibitor" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 275, no. 45, 10 November 2000 (2000-11-10), pages 35122-35128, XP002467834 ISSN: 0021-9258 cited in the application abstract page 35123, left-hand column, line 5 - line 14 page 35128, left-hand column, line 13 - line 42</p>	1-12
Y	<p>US 5 436 392 A (THOMAS JOHN C [US] ET AL) 25 July 1995 (1995-07-25) abstract column 3, line 30 - line 53</p>	1-12
Y	<p>WO 01/20008 A (UNIV LEIDEN [NL]; HOGE MEPELINK ANNEKE HF [NL]; SCARPELLA ENRICO [NL]) 22 March 2001 (2001-03-22) page 4, line 1 - line 4 page 8, line 14 - line 16 claim 7</p>	1-12
Y	<p>JIANG HAOBO ET AL: "Organization of serpin gene-1 from Manduca sexta: Evolution of a family of alternate exons encoding the reactive site loop" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 45, 1996, pages 28017-28023, XP002513184 ISSN: 0021-9258 abstract figure 2 figure 7A</p>	1-12
Y	<p>JIANG H ET AL: "MUTUALLY EXCLUSIVE EXON USE AND REACTIVE CENTER DIVERSITY IN INSECTSERPINS" JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY OF BIOCHEMICAL BIOLOGISTS, BIRMINGHAM,; US, vol. 269, no. 1, 1 January 1994 (1994-01-01), pages 55-58, XP002057503 ISSN: 0021-9258 abstract figure 1 figure 3</p>	1-12

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2008/064728

Patent document cited in search report		Publication date		Patent family member(s)	Publication date
US 5436392	A	25-07-1995	US	5196304 A	23-03-1993
WO 0120008	A	22-03-2001	AU	5824400 A	17-04-2001