# SEED PROTEINASE INHIBITORS FROM ANNUAL MEDICS ACTIVE AGAINST INSECT PESTS

M. Odoardi[1], A. Tava[1] and F. Ceciliani[2]

[1]Istituto Sperimentale Colture Foraggere, viale Piacenza 29, I-20075 Lodi, Italy,

[2] Istituto di Biochimica, Università di Milano, via Celoria 10, I-20133 Milano, Italy

**ABSTRACT**

Several different species of annual medics have been tested for the occurrence and concentration of trypsin inhibitors in the seed. Trypsin Inhibitory Activity (TIA) has been in fact related to the level of resistance to biotic and abiotic stresses. Wide variation was found for TIA among species, with the highest values shown by the snail medic (*Medicago scutellata)*. After a simple and rapid procedure of chromatographic purification, the Trypsin Inhibitor (TI) from snail medic has been tested towards the trypsin-like proteinases extracted from larvae of different phytophagous insects, showing high levels of Inhibitory Activity. The complete amino acid sequence of the snail medic TI consists of 62 residues, corresponding to a relative molecular weight of 6925. Sequence comparison shows significant similarity to other proteins belonging to the Bowman-Birk trypsin inhibitory family, and the closest identity (81%) with the wound-induced trypsin inhibitor from *M. sativa* leaves.

**KEYWORDS**

Protease inhibitors, natural plant defense, insect resistance, annual medics.

**INTRODUCTION**

Widely distributed in the plant and animal kingdom, proteinase inhibitors are low molecular weight proteins that can repress the activity of proteinases by binding into the active site of the enzyme. High concentrations of proteinase inhibitors are found in plant tissues particularly vulnerable to foreign proteases such as seeds, tubers, bulbs and bark (Ryan, 1981). Proteinase inhibitors have been reported as insect pest resistance factors that act by repressing the activity of proteinases in the digestive tract of feeding insect larvae, so retarding development (Mc Manus et al., 1993). The presence, however, of proteinase inhibitors in legume seeds used as food or feed and their potential toxic nature is regarded as a major fact contributing to their decreased nutritional value. Nevertheless, high levels of such compounds in forage and pasture plants seems able to control the insect attack and overcome the use of chemical insecticides. The presence and activity of Trypsin Inhibitors (TI) in seeds of *Medicago sativa* populations and accessions have been previously screened (Odoardi et al., 1991; 1994). The results here presented are focussed on the characterisation of TI from seed of annual medics, with special emphasis to the properties of the snail medic TI.

**MATERIALS AND METHODS**

Seeds of eleven accessions belonging to seven different species of annual medics were utilized. The different strains tested are listed in Table 1. Total soluble proteins have been extracted from seed meal and tested for the Trypsin Inhibitory Activity (TIA) using BApNA as chromogenic substrate, measuring the change in absorbance at 405 nm (Kassel, 1970). One Inhibitory Unit (IU) is defined as the amount of inhibitor which inhibits 1 mg of trypsin. Purification of the snail medic TI followed successive steps: ion exchange chromatography, then gel filtration in an FPLC apparatus. After reduction and carboxymethylation, the TI was sequenced using an Applied Biosystem Sequencer. The amino acid composition after gas phase hydrolysis was performed using a JASCO HPLC. Similarities between the primary structure of the snail medic TI and other proteins were searched with the Swiss Prot Data Bank. Larval proteinases were extracted from defatted larvae of *Adoxophyes orana, Hyphantria cunea, Lobesia botrana* and *Ostrinia nubilalis* and assayed as trypsin-like crude extract for inhibitory activity (Kassel, 1970).

**RESULTS AND DISCUSSION**

A well detectable Trypsin Inhibitory Activity (TIA) has always been observed in all the seed samples analyzed. Previous surveys on the alfalfa trypsin inhibitor content in seeds of *M. sativa* strains and cultivars showed wide variation for this seed protein fraction (Odoardi et al., 1991; 1994). A wider range of variation has been detected in the present investigation, with significant differences among the species here studied. Table 1 shows the TIA values expressed as IU per g of seed. The annual species showed on the whole high TIA levels, with a mean value of 1.41 IU, compared to 0.58 as the mean value found in the *M. sativa* complex (Odoardi et al., 1994). *M. scutellata*, the snail medic, showed the highest TIA (2.80 IU), and *M. tornata* the lowest one (0.38 IU). It should be noted that the snail medics are the cultivars with the largest seed size among annual and perennial medics (Piano and Francis, 1992). To some extent, the level of TIA seems to be related to the seed size and the storage proteins per seed and, therefore, to the necessity to protect highly appetizing seeds from insects and other pests. The presence of a particularly interesting TI in *M. scutellata*, which showed the highest activity in the seed, together with the highest level of insect resistance in the field (Sorensen et al., 1988), stimulated further studies for the characterisation of this protein. The purification procedure of the crude seed extract allowed to obtain a highly purified protein in few steps. After pH adjustment using acetate buffer (pH 3.8) the crude seed extract was purified by three subsequent chromatographic steps. The primary structure of the purified inhibitor was determined after reduction and carboxymethylation of the protein with automated amino-terminal analysis, allowing the identification of the first 44 residues. The complete sequence was determined after characterisation and sequence alignment of all the peptides obtained by tryptic digestion of the snail medic TI. Its complete amino acid sequence is shown that the protein consists of 62 residues, corresponding to a molecular mass of 6925. The snail medic TI is the first serine-proteinase inhibitor to be isolated from seeds of a *Medicago* species; a comparison of its primary structure with those in the Swiss Prot Protein Data Bank revealed that this protein belongs to the Bowman-Birk inhibitor family, with the highest degree of identity (81 %) versus the wound-induced alfalfa trypsin inhibitor (ATI) from *M. sativa* leaves (Brown et al., 1985). The snail medic TI was also assayed *in vitro* against proteases from four species of insect pests belonging to the order Lepidoptera, to assess the general hypothesis that serine proteinase inhibitors have the potential to protect plants against herbivorous insects. Phytophagous Lepidoptera have been used as model insects, because they primarily use serine proteinases for proteolytic digestion. The larval digestive enzymes extracted from the four insect species resulted trypsin-like enzymes, and were clearly inhibited by the snail medic TI, as reported in table 2. In conclusion, additional investigations are needed to assess a possible use of this type of inhibitor as a tool to improve plant resistance to insect attack. In any cases, the introduction of proteinase inhibitor genes also into forage legumes using genetic engineering will lead to agronomically important fodder species resistant to insect attack, and stimulate more investigations on these molecules. Purification and characterization of such trypsin inhibitors could give

valuable results in order to enhance natural defence mechanisms against plant pests in cultivated alfalfa.

**ACKNOWLEDGEMENTS**
Research supported by the Italian Ministry of Agriculture, Food and Forestry Resources, in the framework of the Project "Resistenze genetiche delle piante agrarie agli stress biotici e abiotici".

**REFERENCES**
**Brown, W.E, K. Takio, K. Titani and C.A. Ryan.** 1985. Wound-induced Trypsin Inhibitor in alfalfa leaves: identity as a member of the Bowman-Birk inhibitor family. Biochemistry **24:** 2104-2108.
**Kassel, B.** 1970. A trypsin inhibitor from barley. Pages 839-871 *in* S.P. Collowick and N.O. Kaplan, eds. Methods in Enzymology (vol.19) Academic Press, New York, N.Y.
**McManus, M.T., R.W. Scott, M. Tasneem, D.W.R. White, P.G. McGregor and B.L. Barker.** 1993. Proteinase inhibitors: evaluation as insect pest resistance factors in transgenic plants. Proc. 17th Int. Grass. Cong., Palmerston North, New Zealand, vol. 2, pp. 1168-1170.
**Odoardi, M., L. Valdicelli, N. Berardo and A. Tava.** 1991. Proteinase inhibitors in *Medicago sativa* populations. Proc. EUCARPIA Medicago sativa group 9th meeting, Szarvas, Hungary, pp. 56-61.
**Odoardi, M., R. Cremona, C. Cunico, L. Pecetti, A. Tava and L. Valdicelli.** 1994. Characterization of trypsin inhibitors in seeds of different *Medicago* species. J. Genet. & Breed. **48:** 377-382.
**Piano, E. and C.M. Francis.** 1992. The annual species of *Medicago* in the Mediterranean region: ecogeography and related aspects of plant introduction and breeding. Proc. 10th Int. Mtg. EUCARPIA Medicago spp Group, Lodi, Italy, pp. 373-385.
**Ryan, C.A.** 1981. Protease inhibitors. Pages 351-370 *in* A. Marcus, ed. The biochemistry of plants (vol. 6) Academic Press, New York, N.Y.
**Sorensen, E.L., R.A. Byers and E.K. Horber.** 1988. Breeding for insect resistance. Pages 859-902 *in* A.A. Hanson, D.K. Barnes and R.R. Hill Jr., eds. Alfalfa and alfalfa improvement (Agronomy monograph n. 29) ASA, CSSA and SSSA, Madison.

**Table 1**
Annual *Medicago* species tested for Trypsin Inhibitory Activity (TIA), expressed as Inhibitory Units (UI) per g of seed.

| Species | Cultivar | TIA/g of seed |
|---|---|---|
| *M. lupulina* | Virgo P. | 1.33 |
| *M. murex* | Orion | 1.48 |
| | Zodiac | 0.75 |
| *M. polymorpha* | Circle Valley | 1.02 |
| | Santiago | 0.51 |
| | Serena | 0.75 |
| *M. rugosa* | Paraponto | 1.82 |
| | Sapo | 1.31 |
| *M. scutellata* | Sava | 2.80 |
| *M. tornata* | Tornafield | 0.38 |
| *M. truncatula* | Ascot | 1.78 |

**Table** 2
Effect of snail medic Trypsin Inhibitor against insect proteases, expressed as % of that against trypsin.

| Protease | % Inhibition |
|---|---|
| Bovine trypsin | 100 |
| *Adoxophyes orana* | 76 |
| *Hyphantria cunea* | 68 |
| *Lobesia botrana* | 72 |
| *Ostrinia nubilalis* | 81 |