Pergamon

PII: S0031-9422(96)00483-9

Phytochemistry, Vol. 44, No. 3, pp. 393–398, 1997

0031-9422/97 \$17.00 + 0.00

A TRYPSIN INHIBITOR FROM SNAIL MEDIC SEEDS ACTIVE AGAINST PEST PROTEASES

FABRIZIO CECILIANI, ALDO TAVA,* RENATO IORI,† MICHELE MORTARINO, MIRIAM ODOARDI* and SEVERINO RONCHI‡

Istituto di Fisiologia Veterinaria e Biochimica, Università di Milano, 10 via Celoria, I-20133 Milano, Italy; *Istituto Sperimentale Colture Foraggere, 29 viale Piacenza, I-20075 Lodi, Italy; †Istituto Sperimentale Colture Industriali, 133 via di Corticella, I-40129 Bologna, Italy

(*Received in revised form* 21 *June* 1996)

Key Word Index—*Medicago scutellata*; Leguminosae; snail medic; serine proteinase inhibitor; insect resistance.

Abstract—A protein trypsin inhibitor from seeds of snail medic (*Medicago scutellata*), MsTI, has been purified by ion-exchange chromatography, gel-filtration chromatography and reverse-phase HPLC. The protein inhibits the catalytic activity of bovine β-trypsin, with an apparent K_d of 1.8×10^{-9}, but exhibits no activity towards bovine α-chymotrypsin. Moreover, MsTI inhibits the trypsin-like proteinase activity present in larvae of the crop pests *Adoxophyes orana, Hyphantria cunea, Lobesia botrana* and *Ostrinia nubilalis*. The complete amino acid sequence of MsTI consists of 62 residues corresponding to a M_r of 6925. Sequence comparison shows that MsTI exhibits significant similarity to other proteins belonging to the Bowman–Birk trypsin inhibitor family, and the closest identity (81%) with the wound-induced trypsin inhibitor from *Medicago sativa* leaves. Copyright © 1997 Elsevier Science Ltd

---

INTRODUCTION

Seeds from Leguminosae contain a number of serine proteinase inhibitors which belong to at least two distinct inhibitor families: the Kunitz soybean trypsin inhibitor and the Bowman–Birk soybean proteinase inhibitor [1, 2].

The possible roles suggested for seed proteinase inhibitors include the control of endogenous proteinases in the metabolism of storage proteins and during seed dormancy [3]. Moreover, serine proteinase inhibitors appear to function as a part of the plant defensive system against pests via inhibition of their proteolytic enzymes [4, 5]. Finally, due to their high content of cysteine residues, serine proteinase inhibitors may also be seen as storage proteins which are degraded during germination and seedling growth [6].

Our aim is the study of the role of plant proteinase inhibitors as protective agents against herbivorous insects. Since the high level of serine proteinase inhibitors in many plants has been related to pest resistance, the presence of these inhibitors is usually considered a desirable trait to increase plant resistance against pathogens. We therefore examined trypsin inhibitory activity in the seeds of some *Medicago* species, including annual and perennial types adapted to grazing, and generally known as insect resistant or tolerant [7]. Persistency of viable seed in the soil, particularly of annual self-reseeding species, is in fact an important characteristic of pasture legumes, spreading in various environmental conditions for their rusticity and increasing their economic value for preventing soil erosion and for the production of organic nitrogen, as well as pasturage during periods of low production from traditional pasture species [8, 9]. Results of a previous study clearly indicate that the snail medic (*Medicago scutellata* L.) seeds exhibit a significantly higher content of trypsin inhibitory activity (TIA) than the other species or cultivars tested [7]. *Medicago scutellata* is presently an economically important source of pasture cultivars in Australia and together with other glandular-haired *Medicago* species is potentially useful as a source of germplasm for improving alfalfa resistance to insects [10].

This paper reports the purification, the amino acid sequence and the inhibitory properties of a trypsin inhibitor from *M. scutellata* seeds (MsTI), its homology with some other serine-proteinase inhibitors, and its inhibitory activity towards trypsin-like proteinases extracted from four species of larval herbivorous Lepidoptera.

RESULTS AND DISCUSSION

The purification procedure of the crude seed extract allowed the production of a protein with high purity grade in a few steps. It is interesting to note that affinity

---

‡Author to whom correspondence should be addressed.

chromatography, usually adopted for serine proteinase inhibitor purification, was in this case omitted. Affinity chromatography can result in breakdown products from immobilized trypsin [11, 12], while high yields of pure native protein are obtained with the reported method. The crude seed extract accounted for a total of 26.6 units of trypsin inhibitory activity (UI) with specific activity of 0.04 UI mg^{-1} of protein. After pH adjustment using acetate buffer, a large amount of precipitate was removed by centrifugation and the specific activity increased to 0.34 UI mg^{-1} of protein. Further purification of the inhibitory activity was performed by the three subsequent chromatographic steps, obtaining overall 8.6 UI of purified inhibitor, as shown by capillary electrophoresis analysis (data not reported) and by Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) analysis reported in Fig. 1.

The primary structure of MsTI was determined after reduction and carboxymethylation of the protein. Automated amino-terminal analysis of reduced and carboxymethylated MsTI allowed the identification of the first 44 residues. The complete MsTI sequence was determined after characterization and sequence alignment of all the peptides obtained by tryptic digestion of MsTI. MsTI (10 nmol) was digested with trypsin. Tryptic peptides were purified on a RP-HPLC, as described in the Experimental section. All fractions were tested for homogeneity by capillary electrophoresis: fractions showing more than one peptide were further purified using different conditions (data not reported). Finally, all the pure peptides were sequenced. Figure 2 summarizes the data used to determine the complete primary structure of MsTI: the protein consists of 62 residues, and the calculated M_r of 6925 is very close to the value of 6926 obtained by MALDI-TOF mass spectrometry (Fig. 1). Moreover, the amino acid composition deduced from the sequence (data not reported) agrees well with that determined experimentally. No indication of heterogeneity was noted during sequencing.

The inhibitory properties of MsTI are presented in Fig. 3, which shows the isotherm for MsTI binding to trypsin using benzoyl-D,L-arginine *p*-nitroanilide (BApNA) as substrate, at pH 8 and 21°. MsTI is a strong inhibitor of trypsin, since the apparent K_d for the formation of the trypsin: MsTI complex is 1.8×10^{-9} M, but exhibits no measurable inhibitory activity toward chymotrypsin.

MsTI is the first serine-proteinase inhibitor to be isolated from seeds of a *Medicago* species. As already reported, the complete amino acid sequence of the protein consists of 62 residues: a comparison of the primary structure of MsTI with those in the SwissProt Protein Data Bank revealed that this protein belongs to the Bowman–Birk inhibitor family. Figure 4 shows the comparison of the primary structure of MsTI with that of other Bowman–Birk inhibitors purified from Leguminosae. Figure 5 shows the identity degree calculated after this comparison. As expected, MsTI exhibits the highest degree of identity (81%) vs the wound-induced alfalfa trypsin inhibitor (ATI) from *Medicago sativa* leaves [13].

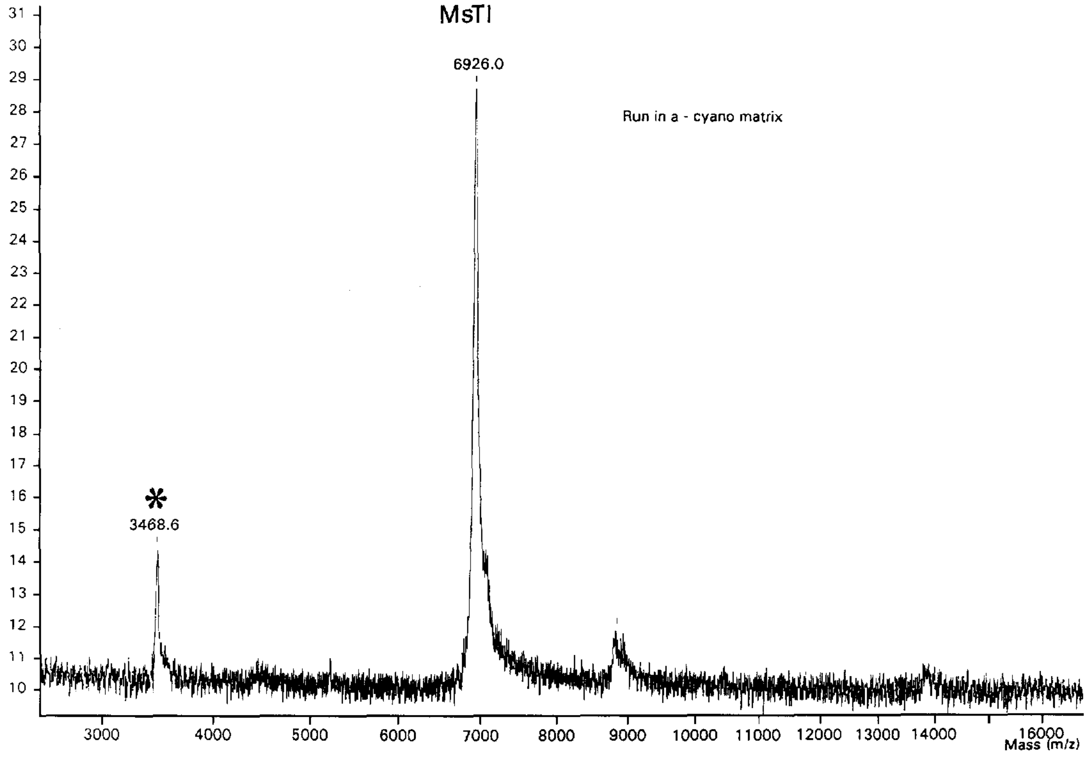

Fig. 1. Mass spectrum of MsTI after RP-HPLC. See text for details. *Indicates double charge ions.

```
                                                                                    20
Thr Lys Ser Thr Thr Thr Ala Cys Cys Asp Phe Cys Pro Cys Thr Arg Ser Ile Pro Pro
———————————————————————————————— N-ter ————————————————————————————————————————
← 1 →  ← — — — — — — — — — — 2 — — — — — — — — — →  ← — 3 — —

                                                                                    40
Gln Cys Gln Cys Thr Asp Val Arg Glu Lys Cys His Ser Ala Cys Lys Ser Cys Leu Cys
———————————————————————————————— N-ter ————————————————————————————————————————
— — 3 — — — — — — — — →  ← 4 →  ← — — 5 — — → ← — — 6 — — —

                                                                                    60
Thr Lys Ser Ile Pro Pro Gln Cys Arg Cys Tyr Asp Ile Thr Asp Phe Cys Tyr Pro Ser
———————
— — →  ← — — — 7 — — — →  ← — — — — — — — 8 — — — — — — — —

     62
Cys Arg

— — →
```

Fig. 2. The primary structure of MsTI. The continuous line indicates the sequence derived from amino-terminal sequencing of the entire protein. The dashed line indicates the sequence derived from tryptic digestion.

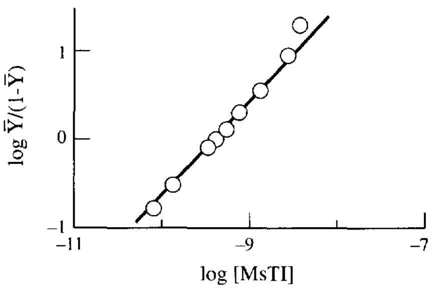

Fig. 3. Amount of MsTI bound to trypsin (○) as a function of the free inhibitor concentration, expressed in M. Values of K_d were obtained as described in ref. [12].

The inhibitory activities of the MsTI and the wound-induced alfalfa trypsin inhibitor are similar, since our value of K_d for the formation of serine-proteinase complex is in good agreement with that observed for ATI binding for trypsin ($K_d = 1 \times 10^{-10}$ M). The Bowman–Birk type inhibitors from Leguminosae are known to be double-headed, with two different sites, one for each of the two structural domains, which interact with the proteinase independently [14].

Figure 6 shows a graphic representation of the primary structure of MsTI, including the seven disulphide bridge positions and the two P1 reactive sites, as suggested by homology comparison. Since the duplicated P1 sites contain Arg and Lys at position 16

```
 1                 TKSTTTA-CCDFCPCTRSIPPQCQCTDVR-EKCHSACKSCLCTKSIPPQCRCYDITDFCYPSCR          62
 2                     TTA-CCNFCPCTRSIPPQCRCTDIG-ETCHSACKTCLCTKSIPPQCHCADITNFCYPKCN          58
 3                 GDDVKSA-CCDTCLCTRSQPPTCRCVDVG-ERCHSACNHCVCNYSNPPQCQCFDTHKFCYKACHSSEKEEVIKN 72
 4                 GDDVKSA-CCDTCLCTKSEPPTCRCVDVG-ERCHSACNSCVCRYSNPPKCQCFDTHKFCYKSCHN         63
 5        SHGHDETTDEPSESSKPCCDQCSCTKSMPPKCRCSDIRLNSCHSACKSCACTYSIPAKCFCTDINDFCYEPCKSSRDDDWDN 82
 6          DHHHSTDEPSESSKPCCDECACTKSIPPQCRCTDVRLNSCHSACSSCVCTFSIPAQCVCVDMKDFCYAPCKSSHDD   76
 7                  DDESSKPCCDQCACTKSNPPQCRCSDMRLNSCHSACKSCICALSYPAQCFCVDITDFCYEPCKPSEDDKEN   71
 8           HEHSSDESSESSKPCCDLCTCTKSIPPQCHCNDMRLNSCHSACKSCICALSEPAQCFCVDTTDFCYKSCHNNAEKD  76
 9        SVHHQDSSDEPSESSHPCCDLCLCTKSIPPQCQCADIRLDSCHSACKSCMCTRSMPGQCRCLDTHDFCHKPCKSRDKD  78
10              SHDEPSESSEPCCDSCDCTKSKPPQCHCANIRLNSCHSACKSCICTRSMPGKCRCLDTDDFCYKPCESMDKD   72
11     MELNLFKSDHSSSDDESSKPCCDLCMCTASMPPQCHCADIRLNSCHSACDRCACTRSMPGQCRCLDTTDFCYKPCKSSDEDDD 83
12  MCILSFLKSDQSSSYDDDEYSKPCCDLCMCTRSMPPQCSCEDIRLNSCHSDCKSCMCTRSQPGQCRCLDTNDFCYKPCKSRDD    83

Consensus sequence        CC  C CT S PP C C        CHS C  C C  S P  C C C   FC   C
```

Fig. 4. Homology comparison of the primary structure of MsTI with that of other Bowman–Birk inhibitors purified from Leguminosae. Consensus sequence was determined considering the residues that were identical in all 12 sequences (bold upper case). (1) MsTI (*Medicago scutellata* trypsin inhibitor). (2) Bowman–Birk type wound induced trypsin inhibitor, *Medicago sativa*. (3) Bowman–Birk type proteinase inhibitor (VAI), *Vicia angustifolia* (common vetch). (4) Bowman–Birk type proteinase inhibitors, *Phaseolus vulgaris* (adzuki bean) (*Vigna angularis*). (5) Bowman–Birk type proteinase inhibitor (FBI), *Vicia faba* (bean). (6) Bowman–Birk type proteinase inhibitor DE-3, *Dolichos axillaris* (*Macrotyloma axillare*). (7) Bowman–Birk type proteinase inhibitor (BBI), *Glycine max* (soybean). (8) Bowman–Birk type proteinase inhibitor DE-4, *Dolichos axillaris* (*Macrotyloma axillare*). (9) Bowman–Birk type proteinase inhibitor I-A, I-B, and I-A′, *Phaseolus angularis* (adzuki bean) (*Vigna angularis*). (10) Bowman–Birk type proteinase inhibitor, *Phaseolus aureus* (mung bean) (*Vigna radiata*). (11) Bowman–Birk type proteinase inhibitor, C-II, *Glycine max* (soybean). (12) Bowman–Birk type proteinase inhibitor D-II (IV), *Glycine max* (soybean).

	1	2	3	4	5	6	7	8	9	10	11	12
1	100											
2	81	100										
3	55	58	100									
4	55	55	84	100								
5	48	48	43	44	100							
6	53	50	46	44	74	100						
7	50	48	44	43	75	72	100					
8	51	47	43	48	63	67	72	100				
9	51	47	46	44	65	67	61	66	100			
10	47	43	41	43	68	67	61	63	81	100		
11	47	47	46	41	58	61	66	63	71	72	100	
12	50	47	44	41	55	59	63	59	71	68	67	100

Fig. 5. Matrix of the pairwise scores. Values indicate the identity degree between various inhibitors calculated after homology comparison.

and 42, respectively, we can suggest that MsTI belongs to the so called D-II Bowman–Birk Inhibitor Group [1], in which both residues involved in serine-proteinase interaction are Arg or Lys, and which is double-headed for trypsin, but not for chymotrypsin or elastase. As already proposed for the D-II Inhibitor Group [1], MsTI can therefore be viewed as an ancestor of the double-headed serine-proteinase inhibitor. This hypothesis is confirmed by the inhibitory properties, since MsTI can only inhibit trypsin, but not chymotrypsin. MsTI was also assayed *in vitro* against proteases from four species of insect pests—*Adoxophyes orana*, *Hyphantria cunea*, *Lobesia botrana* and *Ostrinia nubilalis*—belonging to the order Lepidoptera, to assess the general hypothesis that serine proteinase inhibitors have the potential to protect plants against herbivorous insects [15]. Phytophagous Lepidoptera have been used as the model insects, because they primarily use serine proteinases for proteolytic digestion [16]. The larval digestive enzymes extracted from the four insect species were trypsin-like enzymes, and were clearly inhibited by the MsTI. The inhibitory activity of MsTI against insect proteases, expressed as % of that against trypsin, ranged from 68 to 81% for *H. cunea* and *O. nubilalis*, respectively.

Other important insect pests whose enzymes are known to differ from trypsin may not be inhibited by MsTI, and additional investigations are needed to assess a possible use of this type of inhibitor as a tool to improve plant resistance to insect attack. On the other hand, digestive enzymes from other insects might be inhibited *in vitro* by MsTI, and further *in vivo* and *in vitro* experiments must be done to verify the persistency of such positive effects. Even if the inhibition of larval growth of herbivorous insects seems to be restricted to the time of induction of non-susceptible proteinases [17], the production of insect-resistant agricultural crops by transgenically enhancing the level of proteinase inhibitors can be the first step for a successful struggle against insect pests. Nevertheless, a careful consideration of the adaptability of insects to proteinase inhibitors must be considered in characterizing other proteinase inhibitor families, and in seeking other abundant sources of new proteinase inhibitors.

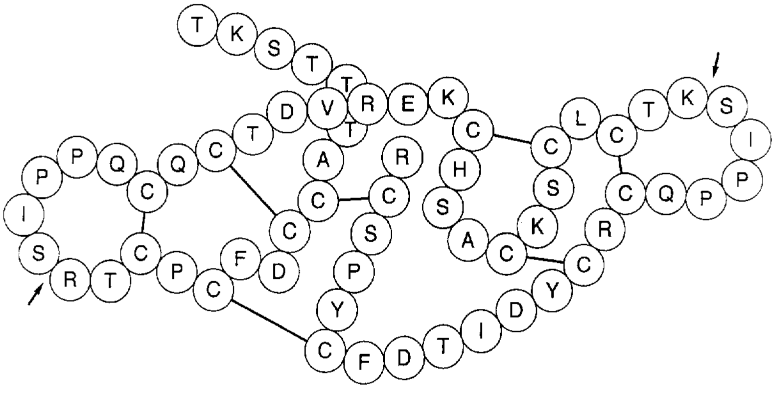

Fig. 6. Graphic representation of primary structure of MsTI. Arrows between Arg16 and Ser17 and Lys42 and Ser43 indicate the probable scissile bond.

Their availability will be useful for additional studies of the pattern of induced proteinases in the modified or adapted insect pests.

EXPERIMENTAL

Plant material. Seeds of the snail medic, *M. scutellata* L. cv. Sava, were kindly provided by Seedco Ltd (Adelaide, Australia). Sequence grade trypsin and all other chemicals used were of the highest purity commercially available.

Purification procedure of MsTI. Snail medic seed (50 g) were defatted by grinding twice in *n*-hexane (1:5 w/v). The organic solvent was removed by filtration and the defatted seed flour was dried under vacuum. Defatted seed flour (10 g) was homogenized with 100 ml of 0.05 M NaCl in an Ultra-Turrax for 5 min. The insoluble material was removed by centrifugation at 10 000 *g*, then re-extracted 2× more with 100 ml of the same soln. The 3 extracts were pooled and the soln was diluted to 500 ml using 0.05 M NaOAc buffer, pH 3.8 (starting buffer). The final pH of the soln was exactly adjusted to 3.8 using HOAc. The ppt. was removed by centrifugation at 15 400 *g* and the clear supernatant was loaded onto a column (2.6 × 10 cm) packed with S-Sepharose Fast Flow and equilibrated with starting buffer. Protease inhibitor was retained quantitatively in the column, washed using starting buffer and eluted with 0.5 M NaCl in the same buffer. The active frs from the first chromatographic step were dialysed thoroughly against starting buffer to remove NaCl, and loaded onto a HR 16/10 prep. column packed with SP-Sepharose H.P. and connected to an FPLC apparatus. The column was equilibrated using the starting buffer and the inhibitor was eluted using a linear concn gradient of 0.0–1.0 M NaCl in the same buffer system. The active frs were pooled and dialysed against 0.01 M NaCl for gel-filtration chromatography, carried out with Superdex 75 Hi Load 26/70 equilibrated with 0.05 M NaPi buffer pH 6.8 containing 0.15 M NaCl. The active frs from gel-filtration were dialysed against 0.01 M NaCl and lyophilized.

Protein determination. Protein concn of all the active fractions from chromatographic steps was determined by the Coomassie blue dye binding method [18]. BSA (1 mg ml^{-1}) was used as standard protein. Protein concn of purified MsTI was estimated by amino acid analysis.

Purification of larval enzymes. Defatted larvae of *A. orana* (Summer fruit tortrix moth), *H. cunea* (tiger moth), *L. botrana* (European graph moth) and *O. nubilalis* (European corn borer) were extracted with 0.05 M NaOAc buffer (pH 3.8) containing 10% (v/v) glycerol, 0.02 M DTT, 0.01 M $MgCl_2$, 0.02 M EDTA to which 5% (wt/larval wt) PVP was added. The ratio of extraction was 1:10 (w/v). In all cases the suspensions were centrifuged at 10 000 *g* for 20 min at 4°. This supernatant served as the source of larval proteinases.

Enzyme inhibition assay. The inhibition of trypsin and trypsin-like activities from insect larvae were followed by monitoring the rate of hydrolysis of BApNA in 0.2 M triethanolamine–HCl buffer (pH 7.8) at 30° measured by following the change in *A* at 405 nm [19]. Chymotrypsin inhibition was assayed using benzoyl-L-tyrosine ethyl ester (BTEE) as substrate, as described in ref. [20]. One unit of inhibitor was defined as the amount of inhibitor which completely inhibited 1 mg of trypsin.

Determination of the apparent equilibrium constant. The apparent dissociation equilibrium constant (K_d) for the binding of MsTI to trypsin and chymotrypsin was determined by measuring its inhibition on the protease activity using Bz-L-Arg-pNA and Bz-L-Tyr-pNA as substrate, respectively [12].

Structural characterization of MsTI. Capillary electrophoresis. Capillary electrophoresis analysis of MsTI and of peptides deriving from its tryptic digestion was performed using a capillary electrophoresis apparatus (Applied Biosystems Model 270 HT) fitted with a 72 cm × 50 μm I.D. (50 cm to the detector). Free soln electrophoresis of MsTI was performed after precoating the capillary with Microcoat™, at 30 kV (30 μA) for 15 min using 40 mM NaOAc (pH 4) as electrolyte. Free soln electrophoresis of peptides was performed at 20 kV (25 μA) for 12 min, using 10 mM Na citrate (pH 2.5). The capillary oven temp. was maintained in both cases at 30°. The electrophoretic mobility of peptides and whole protein was monitored at 220 nm.

Amino acid analysis. Gas-phase hydrolysis was carried out in 6 M HCl 1% (v/v) phenol, at 110° for 24 hr *in vacuo*. Amino acid analysis was performed after pre-column derivatization with 6-aminoquinolyl-*N*-hydroxysuccimidyl carbamate [21] using a JASCO HPLC equipped with 820- FP detector.

Determination of the M_r. M_r of MsTI was determined by MALDI-TOF MS using α-cyano-4-hydroxycinnamic acid matrix (satd soln in 0.1% TFA, 35% MeCN, v/v).

Determination of the primary structure of MsTI. MsTI was reduced and carboxymethylated as described in ref. [22]. Tryptic digestion of MsTI was carried out in 0.1 M Tris–HCl, 1 M guanidine–HCl, (pH 8.5), at 37° for 4 hr, with an enzyme–substrate ratio of 1:25. Enzyme digestion was stopped by lowering the pH to 2 by addition of TFA, and the products were sepd by RP-HPLC on an Aquapore RP-300 C8 column (25 × 0.46 cm). The solvent system used was (A) 0.1% (v/v) TFA in H_2O; (B) 0.075% (v/v) TFA in MeCN; peptides were eluted with a linear gradient from 0 to 50% B in A in 70 min at a flow rate of 0.7 ml min^{-1}. The elution of peptides was monitored at 220 nm. Aliquots from each fr. were analysed for homogeneity by capillary electrophoresis. Frs containing more than one peptide were further purified using a gradient from 0 to 75% MeCN in 5mM NaPi (pH 6) in 75 min. The whole MsTI and the peptides found to be pure were finally sequenced using a Mod. 477/A (Applied Biosystems) sequencer.

Computer sequence analysis. Similarities between the primary structure of MsTI and other proteins were searched with the SwissProt Protein Data Bank.

Acknowledgement—This research was supported by the Italian Ministry of Agriculture, Food and Forestry, National Project "Resistenze genetiche delle piante agrarie agli stress biotici e abiotici".

REFERENCES

1. Laskowski, M. Jr and Kato, I. (1980) *Ann. Rev. Biochem.* **49**, 593.
2. Birk, Y. (1994) in *Bioactive Substances in Food of Plant Origin* (Kozlowska, H., Fornal J. and Zdunczyk Z., eds), p. 202.
3. Richardson, M. (1981) *Food Chem.* **6**, 235.
4. Gustafson, G. and Ryan, A. C. (1971) *J. Biol. Chem.* **251**, 279.
5. Ryan, A. C. (1990) *Ann. Rev. Phytopathol.* **28**, 425.
6. Papastoitsis, G. and Wilson, K. A. (1991) *Plant Physiol.* **96**, 1086.
7. Odoardi, M., Cremona, R., Cunico, C., Pecetti, L., Tava, A. and Valdicelli, L. (1994) *J. Genet. Breed.* **48**, 377.
8. Piano, E. and Francis, C. M. (1992) *Proc. X Int. Confer. Eucarpia Medicago spp.* p. 373. Lodi.
9. Brummer, E. C., Bouton, J. H. and Kochert, G. (1995) *Genome* **38**, 362.
10. Sorenson, E. L., Byers, R. A. and Horber, E. K. (1988) in *Alfalfa and Alfalfa Improvement* (Hanson, A. A., Barnes, D. K. and Hill, R. R., eds), p. 859. American Society of Agronomy, Madison, WI.
11. Wilimowska-Pelc, A. (1989) *Acta Soc. Botanica Pol.* **58**(4), 563.
12. Ceciliani, F., Bortolotti, F., Menegatti, E., Ronchi, S., Ascenzi, P. and Palmieri S. (1994) *FEBS Letters* **342**, 221.
13. Brown, W. E., Takio, K., Titani, K. and Ryan, A. C. (1985) *Biochemistry* **24**, 2105.
14. Ikenaka, T. and Norioka, S. (1986) in *Proteinase Inhibitor* (Barret, A. S. and Salvesen, G., eds), p. 361. Elsevier, Amsterdam.
15. Broadway, R. M., Duffey, S. S., Pearce, G. and Ryan, A. C. (1986) *Entomologia Exp. Appl.* **41**, 33.
16. Christeller, J. T., Laing, W. A., Markwick, N. P. and Burgess, E. P. J. (1992) *Insect Biochem. Molec. Biol.* **22**, 735.
17. Broadway, R. M. (1995) *J. Insect Physiol.* **41**, 107.
18. Bradford, M. M. (1976) *Analyt. Biochem.* **72**, 248.
19. Kassel, B. (1970) *Meth. Enzymol.* **19**, 839.
20. Hummel, B. C. W. (1959) *Can. J. Biochem. Physiol.* **37**, 1393.
21. Cohen, S. and Michaud, D. P. (1993) *Analyt. Biochem.* **211**, 279.
22. Swenson, R. P., Williams, C. H. Jr, Massey, V., Ronchi, S., Minchiotti, L., Galliano, M. and Curti, B. (1982) *J. Biol. Chem.* **257**, 8817.