Isolation and Characterization of a Trypsin Inhibitor from White Mustard (*Sinapis alba* L.)

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A new trypsin inhibitor has been isolated in high yield by affinity chromatography from bulk of the soluble seed proteins of white mustard. The inhibitor is a protein with a molecular weight of 18000 ± 1000 daltons and has 142 ± 5 amino acid residues with a relatively high content of serine, lysine, glycine, and aspartic acid (12–13, 13–15, 15, 11–12 residues, respectively) residues. The mustard trypsin inhibitor inhibits bovine β-trypsin strongly and specifically and shows very little or no activity toward bovine α-chymotrypsin, thrombin, kallikrein, and human urokinase. The stoichiometry of the trypsin inhibitor complex is 1 to 1, with a dissociation constant of 2.2×10^{-9} M. UV absorption, circular dichroic, and fluorescence properties have been investigated at room temperature as a function of pH. Several analogies with the soybean trypsin inhibitor (Kunitz) have been noticed, particularly in the circular dichroic spectra.

INTRODUCTION

Proteinase inhibitors are widespread in the plant kingdom and they are found particularly in seeds of graminaceae and leguminosae and in tubers of solanaceae. Of the several possible roles suggested for proteinase inhibitors, the prevailing ones seem to be the control of endogenous proteinases during seed dormancy and protection against the proteolytic enzymes of many parasites. The last mentioned function appears to be the most important for the improvement of plant resistance to microbial and insect damage (Green and Ryan, 1972).

The molecular properties of most plant proteinase inhibitors have been well studied (Ryan, 1981; Richardson, 1981) and they seem to be rather similar. Generally these inhibitors have a single polypeptide chain of low molecular weight which contains a high percentage of S–S cross-links. In turn, this brings about stability toward thermal and pH denaturation. As far as we know, trypsin inhibitors in cruciferae, and white mustard seeds in particular, have never been isolated, although they would have important implications (i) in the metabolism of storage proteins, (ii) as protective agent against insect attacks, and (iii) in human and animal nutrition. The present paper describes the purification and preliminary characterization of mustard trypsin inhibitor (MTI) from white mustard seeds.

This paper should also be seen as a continuation of our work with sunflower seeds (Walde et al., 1984). From the source we were able to isolate several proteases and we also noticed the presence of a weak trypsin inhibitor, although attempts to isolate it proved to be very difficult, possibly because of the presence of chlorogenic acid. These studies prompted our interest in looking for the presence of trypsin inhibitors in other oil seeds of agricultural interest in which the isolation procedure would be easier. Mustard seeds happened to be a very fortunate choice.

MATERIALS AND METHODS

Materials. A commercial variety of white mustard seeds (*sinapis alba*) cv. Albatros were obtained from the SIS Foraggera, Bologna. Bovine β-trypsin (TRL 3X crystallized, salt-free) and bovine α-chymotrypsin were supplied by Worthington Chemical Co.; bovine trombin and *N*-α-benzoyl-D,L-arginine-*p*-nitroanilide (BAPNA) were obtained from Sigma Chemical Co. Bovine serum albumin, ovalbumin, chymotrypsinogen, ribonuclease A, Sepharose 4B, and Sephadex G-50 were products of Pharmacia Fine Chemicals, while CMC-Cellulose (CM-52) was obtained from Whatman. Both forms of human urokinase (molecular weights 33000 and 54000 dalton) were kindly provided by Serono S.p.A. Under all the experimental conditions the M_r 33000 and 54000 species of human urokinase show no differences in kinetic, spectral, or binding properties (Ascenzi et al., 1982). Porcine pancreatic kallikrein A and B and bovine basic pancreatic trypsin inhibitor (Kunitz inhibitor) were purchased from Bayer AG. The other reagents were of analytical grade.

Preparation of Crude Extract. The mustard seeds were homogenized with distilled water with an Ultra-Turrax mod. T45. The insoluble material was removed by centrifugation at 17700*g*. Ammonium sulfate was added to the supernatant until 20% saturation and the suspension was then filtered through filter paper. The clear filtrate was equilibrated at pH 4.0 by adding dilute H_2SO_4 and the precipitate removed by centrifugation. The supernatant was adjusted to pH 8.0 into NH_4OH to remove the phytate salts. The clear supernatant was saturated with $(NH_4)_2SO_4$ to 60% concentration. The precipitate, separated by centrifugation, was solubilized with 0.1 M triethanolamine buffer pH 7.8 containing 0.1 M NaCl and 0.01 M $CaCl_2$ and dialyzed thoroughly against the same buffer and then centrifuged.

Purification. The crude MTI solution was loaded into a column packed with trypsin-Sepharose 4B prepared as described by Cuatrecasas and Anfinsen (1971). MTI was retained quantitatively in the column and was eluted with 0.2 M KCl, pH 1.8. After concentration and dialysis, the active fractions were applied to a CM-cellulose column (CM-52 Whatman) preequilibrated with 0.05 M acetate buffer pH 5.4 and the inhibitor was eluted with a linear gradient of NaCl. Fractions from the ion-exchange step, which showed high trypsin inhibitory activity, were pooled and concentrated with Amicon UM 10 membrane, applied to a column of Sephadex G-50 SF preequilibrated with 0.1 M acetate buffer pH 5.4, and eluted with the same buffer solution. The fractions were assayed for inhibition activity and those with sufficient activity (>75 TIU), were pooled, concentrated to a few milliliters, and then lyophilized to a constant weight for the following studies.

Electrophoresis Procedures. Electrophoresis was performed according to Davis (1964) by using slabs of 7.6% polyacrylamide gel at pH 8.9. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was carried out

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according to Laemmly (1970) with 0.75-mm thick, 15% polyacrylamide vertical gel slabs. Gels were stained with Coomassie Brilliant Blue R-250 and destained with acetic acid, methanol, and water (1:4:5).

Isoelectric Focusing. The experiments were carried out on polyacrylamide gels prepared by photopolymerization using pH gradients of 3–10 and 8–10, 5% acrylamide concentration, and 3% cross-linkage.

Molecular Weight Determination. For the estimation of molecular weight the sample and marker proteins were solubilized in 10 mM Tris-HCl buffer pH 8.0 containing 1 mM EDTA, 1% SDS, and 5% mercaptoethanol and then heated at 100 °C for 3 min before SDS-PAGE. The molecular weight was also determinated by gel filtration on a Sephadex G-50 SF column eluted with solution of 0.1 M acetic acid and by ultracentrifugation with a Beckman Model E analytical ultracentrifuge with a 0.2 mg/mL MTI concentration in water at 24000 rpm at 27 °C.

Amino Acid Analysis. The lyophylized protein was hydrolyzed in sealed vials under vacuum with 6 N HCl at 110 °C for 24, 48, and 72 hours. After hydrolysis, the sample was dried by vacuum and analyzed with Carlo Erba Model 3A29 amino acid analyzer equipped with cells for high sensitivity according to Devenyl (1968). The tryptophan content was determined spectrophotometrically according to Edelhoc (1967).

Protein Analysis. The concentration of MTI was determined by amino acid analysis. The trypsin concentration was estimated by measuring the absorbance at 280 nm with $E^{1\%}_{1\ cm} = 15.6$ (Quast et al., 1978).

Spectrophotometry. The UV absorption spectra were measured at 25 °C with an Uvikon 820 spectrophotometer, the CD spectra were measured with a Jasco Model J-40AS, and fluorescence with an Aminco-1000 corrected spectrofluorometer. All measurements were performed at room temperature.

Assay Methods. The assay used for determining the activity of MTI was the inhibition of tryptic hydrolysis in 0.2 M triethanolamine buffer pH 7.8 at 25 °C on BAPNA, measured by following the change in absorbance at 405 nm (Kassel, 1970). The activities of bovine α-chymotrypsin, bovine thrombin, porcine pancreatic kallikrein, and human urokinase were measured according to the method of Ascenzi et al. (1982).

Trypsinogen Activation. Trypsinogen was activated with porcine enterokinase (1% by weight) in 10 mM Tris-HCl, 1 mM benzamidine buffer pH 8.0 at 2 °C for 10 h. The activation was carried out until a plateau value was reached.

Dissociation Constant Determination. The dissociation constant (K_d), the binding constant (K_b) and the standard free energy of binding ($-\Delta G° = RT \ln K_b$) for the trypsin–inhibitor complex were calculated from activity measurements at 25 °C by using BAPNA as substrate (Green and Work, 1953).

RESULTS AND DISCUSSION

Purification and Characterization. The trypsin inhibitor was isolated in high yield from the bulk of the soluble seed proteins by affinity chromatography on trypsin-Sepharose 4B. Protein fractions devoided of MTI activity were eluted with the void volume of the column, whereas the inhibitor was obtained by applying an elution buffer pH 1.8 containing 0.2 M KCl. The pooled MTI active fractions, after concentration and dialysis, were further purified by ion-exchange and gel-filtration chromatography (Figure 1 parts a and b). The isolated trypsin inhibitor was pure as judged by SDS-PAGE, isoelectric focusing, gel-filtration chromatography, and analytical ultracentrifugation. This purification procedure allows the recovery of more than 50% of the initial inhibition activity. This was two trypsin inhibitor units per gram of white mustard seed, corresponding to ca. 2 mg of MTI. The inhibitor loosed its activity when heated in 0.02% aqueous solution for 10 min at 60 °C. After standing overnight at room temperature ca. 50% of the activity is recovered.

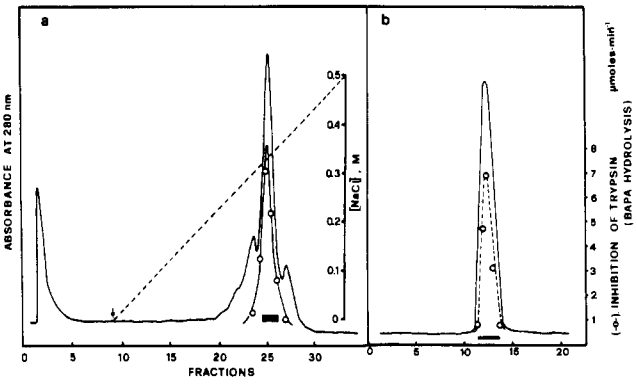

Figure 1. Chromatography on CM-cellulose (1.5 × 12 cm) of pooled active fractions from affinity chromatography. The column, preequilibrated with 0.05 M CH_3COONa pH 5.4, was eluted with the same buffer until fraction 9 (arrow 1) at which point a linear gradient of 0–0.5 NaCl in 0.05 M CH_3COONH_4 (pH 5.4), 75 mL/bottle was applied. Elution was performed at the rate of 20 mL/h in 5-mL fractions. The solid line represents the absorbance at 280 nm, O inhibitor units. Fractions 25 and 26 were pooled and lyophylized. (b) Sephadex G50 superfine gel filtration of the CM-cellulose preparation. Protein (20 mg) was applied to the columnn (1.6 × 90 cm) equilibrated and eluted with 0.1 M CH_3COOH. The solid line represents the absorbance at 280 nm, O specific inhibitory activity.

Table I. Amino Acid Composition of Mustard Trypsin Inhibitor

11–12 Asp	8–9 Pro	5 Tyr	8 Ala
8 Glu	12–13 Ser	7 Phe	10–11 Val
13–15 Lys	7–8 Thr	2 His	8–9 Leu
5 Arg	3 Met	3–4 Trp	15 Gly
	4 Cys		8–9 Ile

The purified inhibitor migrated in SDS polyacrylamide gel as a single polypeptide, of M_r 18400 ± 1000 dalton. The molecular weight was also measured by gel-filtration chromatography on a Sephadex G-75 SF column calibrated with standard proteins, bovine serum albumin (M_r 67000), ovalbumin (M_r 34000), chymotrypsinogen A (M_r 25000), ribonuclease A (M_r 13700), and Kunitz inhibitor (M_r 6500). The molecular weight by gel filtration was estimated to be 19000 ± 1000 dalton. Analytical ultracentrifuge experiments gave a molecular weight of 18700 dalton assuming a partial specific volume of 0.74 $cm^3\ g^{-1}$ (calculated from the amino acid composition).

One should remember at this point that the majority of protease inhibitors isolated from plants have a molecular weight in the range 8000–10000 dalton (Richardson, 1977). The noteworthy exception is the soybean trypsin inhibitor (Kunitz), with 181 amino acid residues and a molecular weight of 21500 dalton (Koide and Ikenaka, 1977). However, similar inhibitors from winged bean, rice, and barley are probably homologous to the soybean inhibitor (Laskowski and Kato, 1980).

The amino acid composition of MTI is shown in Table I. There is still some ambiguity, that we intend to resolve shortly. At the present, we can say that there are 142 ± 5 residues per molecule of MTI, and even in this uncertainty a few interesting peculiarities are apparent. For example, there appear to be four half-cysteine residues which presumably participate in the formation of two

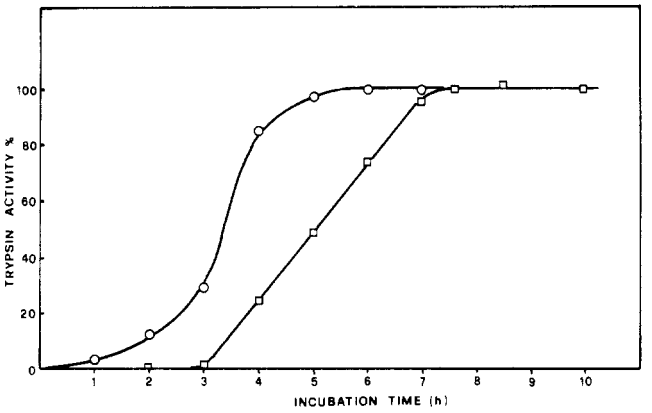

Figure 2. Time course of trypsinogen activation by porcine enterokinase (1% by weight).

disulfide bridges per molecule. Normally, protein trypsin inhibitors isolated from plants have a high S–S content (Ryan, 1981). The soybean trypsin inhibitor has two disulfite bridges. MTI is rich in basic amino acids, particularly in lysine, and in glycine, serine, aspartic acid, and valine. The high content of tryptophan residues appears also to be very characteristic of MTI. In fact, this property distinguishes MTI from the trypsin inhibitors isolated from other plants, including legumes, in which there are normally few tryptophan residues or none (Birk, 1974; Manjunath et al., 1983). The isoelectric point of MTI is around 9.5 or slightly higher (this could not be checked precisely due to limitations of the commercially available ampholine-PAG plates) whereas the pI of the soybean trypsin inhibitor is in the acid pH range (around 4.5).

Specificity. MTI strongly inhibited bovine β-trypsin only and showed very little or no activity toward bovine α-chymotrypsin, bovine thrombin, porcine pancreatic kallikrein, and both forms of human urokinase. Like other plant proteinase inhibitors (Lan et al., 1980), MTI retards the activation of trypsinogen by porcine enterokinase. In the presence of 1.2×10^{-6} M inhibitor full activity of the protein was attained 2 h later (Figure 2).

Studies of the inhibitor activity of MTI indicated that the stoichiometry of the trypsin–inhibitor complex was 1 to 1 (Figure 3). The dissociation constant of the complex, calculated from inhibitory assays in the presence of inhibitor, was 2.2×10^{-9} M and the standard free energy of binding was 11.9 ± 0.5 kcal M^{-1}. The values indicate that the MTI–trypsin complex is remarkably stable, a characteristic also showed by the Kunitz inhibitor trypsin complex.

Spectroscopy. Figure 4 shows the UV absorption spectra of MTI at various pHs. It is a typical protein spectrum, with a maximum at 279 nm, a minimum at 250 nm, and a ratio of the 280/260 nm absorbances of 1.71. Measurements of aqueous neutral solutions gave an apparent extinction coefficient $E_{279nm}(0.1\%)$ of 1.45 ± 0.03. This would correspond to a molar extinction coefficient of 26 126 M^{-1} cm^{-1} assuming a molecular weight of 18 000 dalton. This relatively high value is consistent with the high content of aromatic residues. Changes in the UV spectra are modest in the pH range 2–10, but larger changes take place in the more alkaline range, reflecting the ionization of tyrosine residues rather than a partial denaturation of the protein because the changes are reversible. A shoulder at 295 nm, probably due to the tryptophan contribution, can be observed also in the neutral pH range. Figure 5 parts a and b show the circular dichroic spectra (CD) of MTI in the near and far UV region.

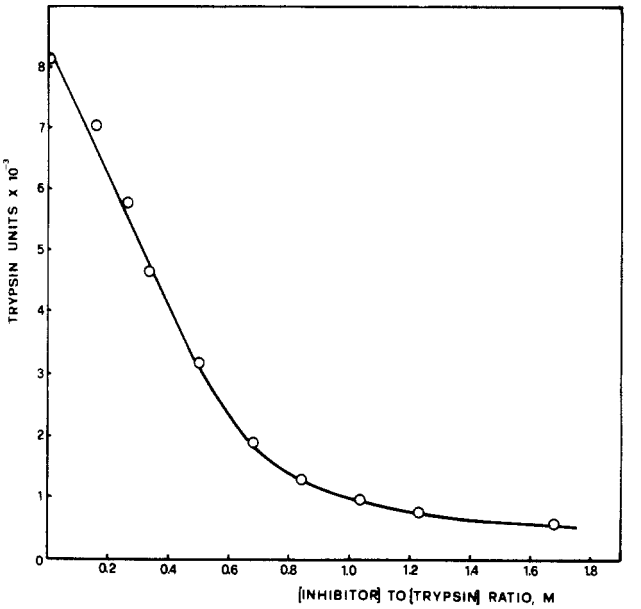

Figure 3. Inhibition of trypsin by MTI as a function of the inhibitor:trypsin molar ratio. Room temperature, pH 7.8, 0.13 M triethanolamine buffer. The trypsin concentration was 0.125 M.

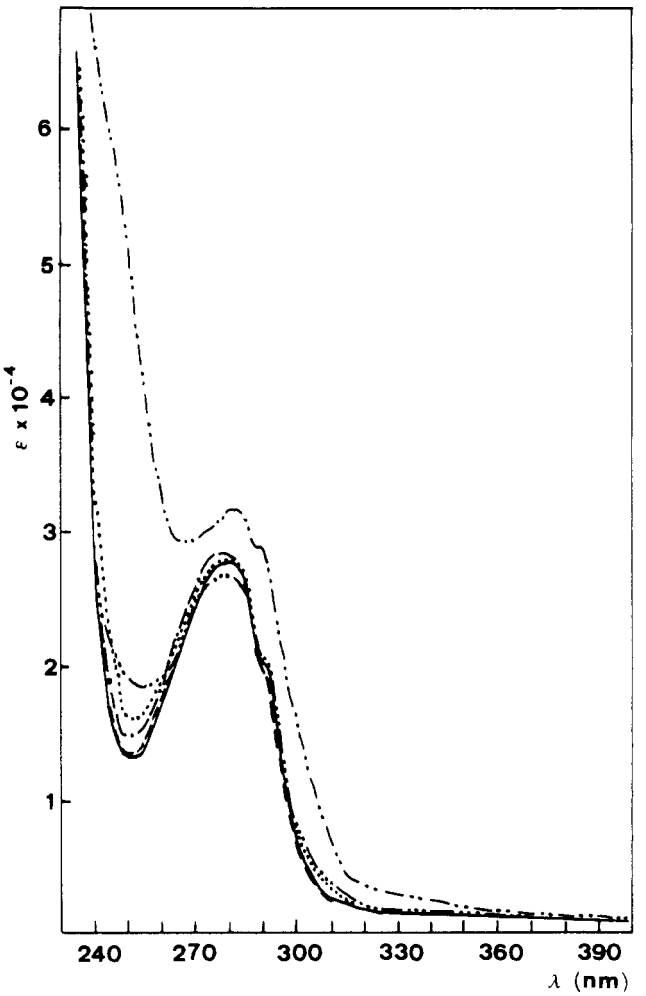

Figure 4. UV absorption spectra vs. pH of aqueous solutions of MTI. Concentration: 0.19 mg MTI/mL. Path length: 1 cm. pH: 2.0 (–·–), 3.6 (––); 4.8 (·); 5.6 (—); 10.1 (–···–); 11.7 (–··–).

Let us consider first the near spectral region in the acid–neutral pH range. The spectrum is typical of simple tryptophan-containing peptides. At extreme acid and

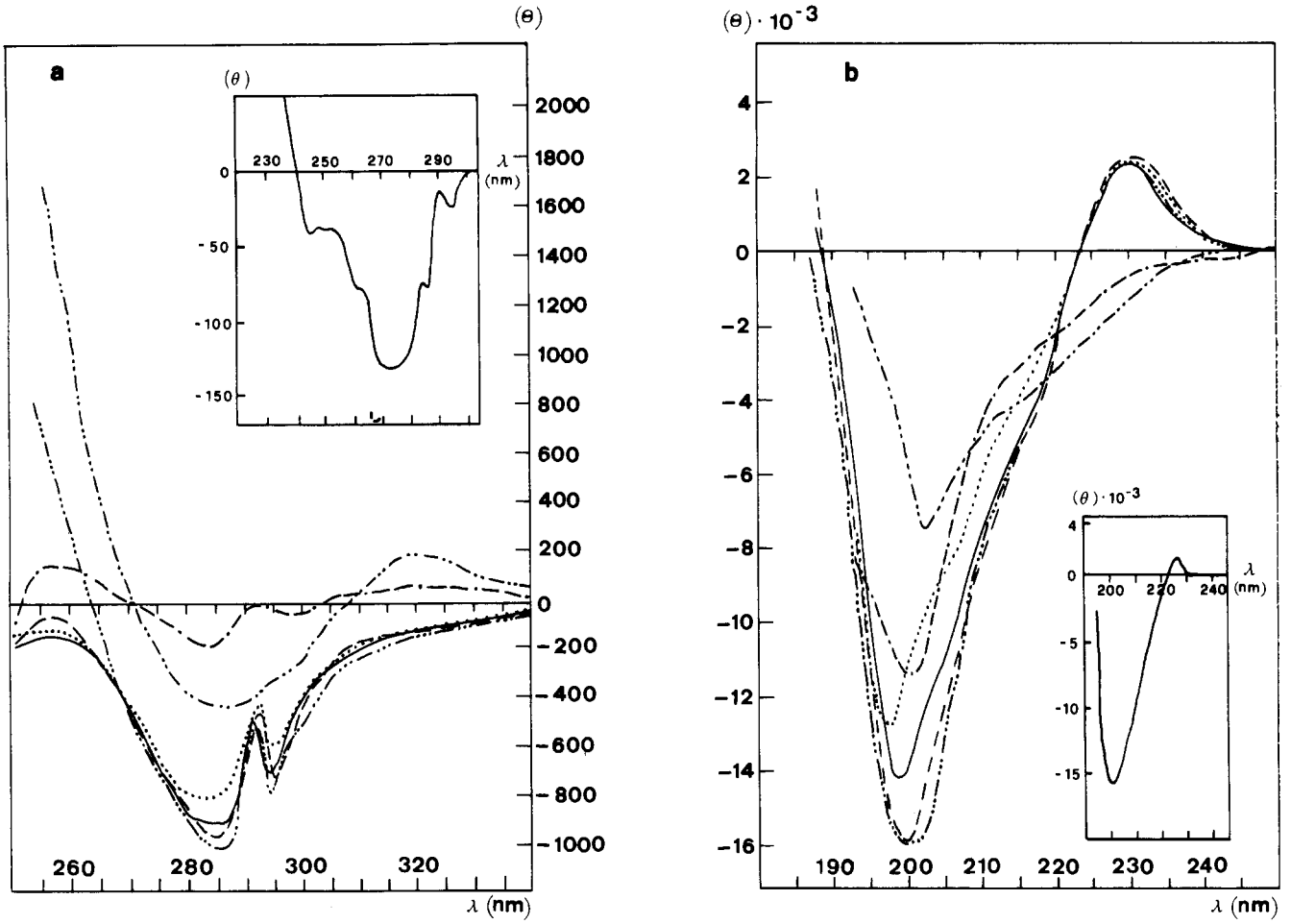

Figure 5. Effect of pH upon CD spectra of aqueous solutions of MTI in the near (a) and far (b) UV range. The pH's are the same as in Figure 4.

alkaline pH values, there are marked changes, which reflect partly the changed UV absorption spectrum (see Figure 4) and partly conformational changes. This last statement is supported also from the onset of the small positive band at 320 nm, which is typical of tryptophan (Rizzo and Luisi, 1977). In conclusion, it appears from Figure 5 part a that the relative orientation of the tryptophanyl residues is not significantly affected by pH changes in the range of pH 3.6 to 10 ca. The near CD spectrum of SBTI is very similar to that of MTI (Jirgensons et al., 1969). Also the appearance of the small positive band at around 250 nm in the alkaline region has been observed in SBTI (Ikeda et al., 1968; Baba et al., 1969). This was attributed to the ionization of tyrosine residues (Baba et al., 1969). As is well-known, it is the far UV range of the CD spectrum which is more diagnostic for the main chain conformation.

As Figure 5 part b shows, the far CD spectrum of MTI is quite similar to that of SBTI (see insert). In fact, both inhibitors are characterized by a strong negative band at 200 nm and by a positive signal in the 225–230-nm region. Since on the basis of conformational studies SBTI is defined a "nonhelical" protein (Jirgensons et al., 1969; Jirgensons, 1978), a finding which is also in agreement with X-ray structure (Sweet et al., 1974; Blow et al., 1974), it is reasonable to suppose a very limited helix content in MTI as well.

The absolute value of the ellipticity at 200 nm for residue of MTI is very close to that of SBTI. The positive band at around 230 nm can be attributed to the contribution of the L_a band of tryptophan (Woody, 1978). As in the case of the near UV CD spectra, dramatic changes are induced by very alkaline pH conditions. Also in the case of SBTI the intensity of the 200-nm band decreases in the pH range 11–12 and this is attended by a decrease of the positive band by 230 nm (Ikeda et al., 1968; Burstein et al., 1973; Toniolo et al., 1978). In the very acid pH range, however, the behavior of MTI and SBTI differs; in the case of SBTI an increase of the intensity of the 200-nm band has been observed on going from neutral pH to pH 2.2–1.0 (Jirgensons, 1973; Toniolo et al., 1978).

The CD spectrum of MTI has also been measured in 50% trifluoroethanol (TFE). In the far UV region a typical helical band was observed at 225 nm, (figure not given) with an intense positive maximum at 195 nm and a negative minimum at 205. Quite similar behavior was observed for SBTI (Tamura and Jirgensons, 1980).

Let us consider now the fluorescence properties of MTI, as recorded with a modified version (Lutz and Luisi, 1983) of the corrected Aminco-1000 spectrofluorometer. For an excitation wavelength in the range 280–295 nm, the emission maximum appears at 340 nm (Figure 6 part a). According to a classification of Burstein et al. (1973), this emission is typical of a protein having tryptophanyl residues at the surface, but with limited interaction with the solvent.

Although the emission properties are dominated by tryptophan, a significant contribution of tyrosine can be determined by the method of Kronman et al. (1971). Furthermore, the excitation spectrum for an emission wavelength of 305 nm gives a λ_{max} 281 nm, as shown in Figure 6 part b, which corresponds to the absorption spectrum of tyrosine.

The pH dependence of the emission was measured in the pH range 2–12. A constant quantum yield was ob-

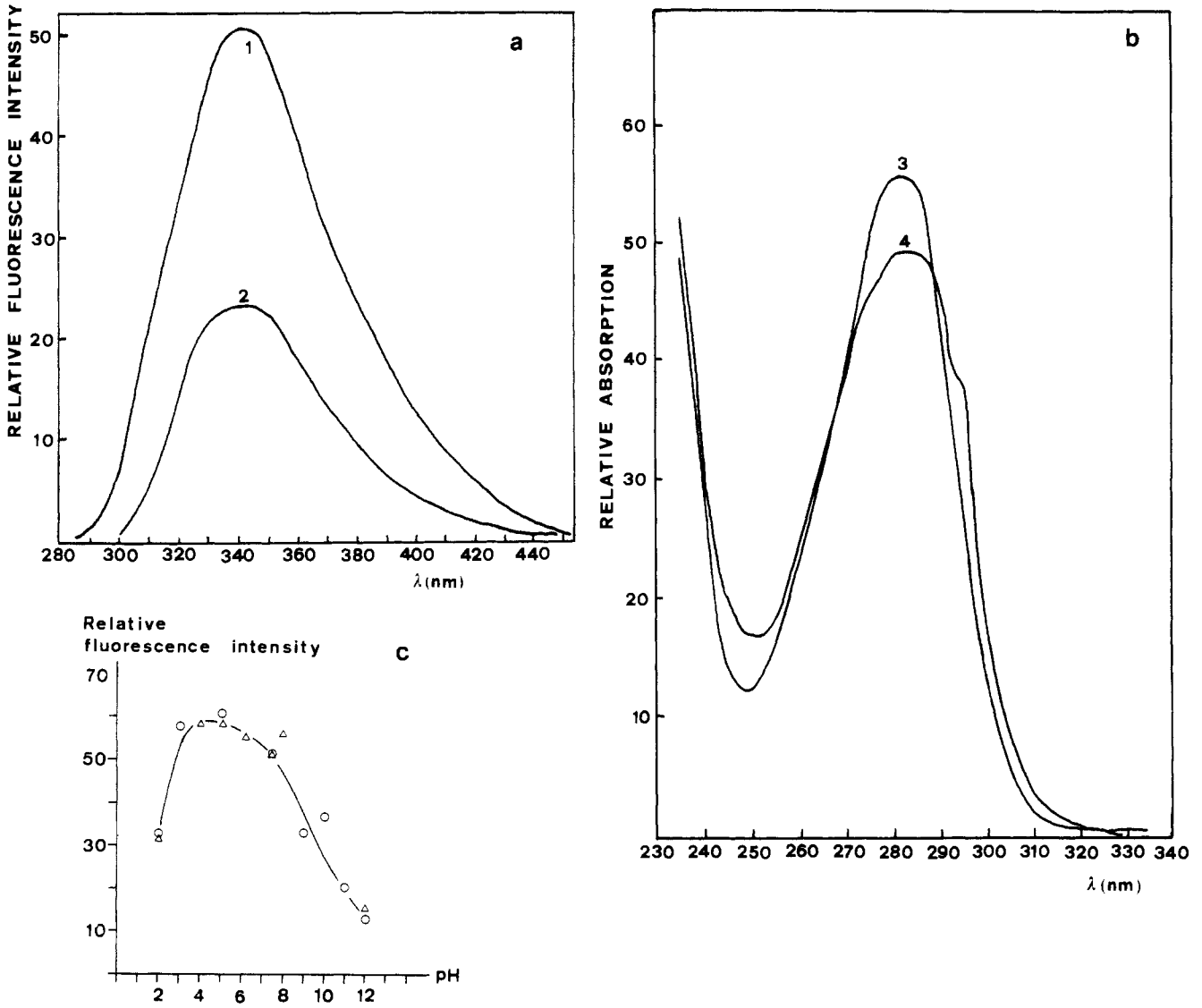

Figure 6. Emission and excitation spectra of aqueous solutions of MTI: (a) emission spectrum after excitation at 280 nm (1) or 295 nm (2); (b) excitation spectra with emission at 305 nm (3) or 340 nm (4); (c) pH dependence of the relative fluorescence intensity of aqueous solution of MTI at 340 nm (λ_{ex} 280 nm).

tained in the pH range 3–9, whereas a sizeable quenching was observed at more acid or at more alkaline pH values (Figure 6 part c). Quenching of the protein fluorescence in the alkaline region is a common feature, whereas the emission behavior in the very acid pH range differs from protein to protein (Steiner and Edelhoch, 1961).

CONCLUDING REMARKS

This first report on MTI is able to shed some light on the basic properties of this new trypsin inhibitor; MTI having about 142 residues belongs to the family of "high molecular weight" protease inhibitors and in this sense seems to be very closely related to SBTI. Another interesting similarity between the two proteins is in the circular dichroic properties. Actually, the spectra of MTI and SBTI are so close to each other that we are tempted to propose the same or a very similar tertiary structure. This similarities in size and folding are surprising in view of the different chemical properties of the two proteins; for example SBTI is an acidic protein, MTI behaves instead as a basic one. The comparison of specificity also reveals significant analogies and differences. The two proteins have about the same affinity toward trypsin. However MTI possesses a higher specificity and actually appears to be the most specific trypsin inhibitor known to date. In view of the easy isolation procedure and of the relatively high yields, MTI may be interesting both for pharmacological applications and to study its influence in human and animal nutrition. The greater the capability of protease inhibitor to block the activity of only one of a group of closely related proteases, the greater is the possible therapeutic value of the substance. Much remains to be done in order to obtain better understanding of the chemical and biological properties of MTI. Classical structural work, e.g., amino acid sequencing and possibly X-ray analysis, are the obvious first objectives also in view to elucidate the physiological role in the plant. In addition, since MTI is able to block also the enterokinase, an enzyme that carries on an important role in the activation of digestive zymogen, its presence could be a significant factor in the protection against seed-eating insects and pathogens that secrete proteolytic enzymes during infection of plant tissues (Ryan, 1981). Therefore, the inhibitor activity data could be useful markers in plant breeding programs aimed to singling out genotypes with high resistance toward parasites.

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Registry No. Trypsin inhibitor, 9035-81-8; trypsin, 9002-07-7; Kunitz, 9088-41-9.

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Composition of the Essential Oil from *Asarum canadense*

Michael G. Motto* and Norman J. Secord

The steam distilled oil obtained from the ground root material of *Asarum canadense* (family Aristolochiaceae) is known in the flavor and fragrance industry as Canadian snakeroot oil (wild ginger). The oil has a spicy odor and flavor and is used in many flavor preparations. We have investigated this essential oil in order to determine its composition. A combination of chromatographic and spectroscopic methods has been used to characterize the oil. We have been able to characterize greater than 90% of the oil and have identified fifty one individual components, of which thirty seven have not previously been reported.

Oil of Canadian snakeroot (wild ginger) is produced by steam distillation of the dried root material of *Asarum canadense* (family Aristolochiaceae). The oil which is chiefly used in flavor preparations, has a strong spicy odor and flavor, the physical properties of which have been described by Guenther (1952). We have undertaken an investigation of this oil in order to determine its composition. Previous investigators, Power and Lees (1902), Ikede et al. (1962), and Bauer et al. (1967) have reported some of the components in this oil. Here now we report a more comprehensive list of the constituents of this essential oil.

EXPERIMENTAL SECTION

The essential oil was obtained by the steam distillation of the finely ground root material of *Asarum canadense*. The total yield of oil obtained in this manner was 3.0%. The individual components of the oil were separated and identified by using the following general scheme.

The crude oil was first distilled under vacuum (0.5 mm) through a column packed with glass coils. The pot temperature was kept at 110 °C. This divided the oil into two general fractions, the distilled material and the pot residue, each constituting about 50% of the oil. Each of these fractions was then further separated in the following manner. The distillate was first fractionated by spinning band distillation under vacuum and then further separated by preparative gas chromatography. The pot residue likewise was further separated by column liquid chromatography, followed by HPLC, and if needed, preparative gas chromatography.

Identification of each of the components in each fraction was first done by GC–MS combined with computer matching of the mass spectrum with that of materials present in our library. Further proof of each component's identification was accomplished by the comparison of the retention index data obtained on two capillary GC columns

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