(Taira, 1983a), foxtail millet *Setaria italica* Beauv. (Taira, 1984), pearl millet *Pennisetum americanum* (L.) K. Shum. (Jellum and Powell, 1971), Job's tears *Coix lacryma-jobi* L. var. *ma-yuen* Stapf (Taira et al., 1985), and corn *Zea mays* L. (Jellum, 1970). Figure 1 shows the relationship between oleic and linoleic acid contents of Indica and Japonica types. The scatter diagram could be divided into Indica and Japonica type groups. In the case of the same oleic acid content between both types, it was shown that Indica type was lower than Japonica type in linoleic acid content. According to the diagram, it is supposed that both groups may be caused by the difference between Indica and Japonica types in heading date or temperature during ripening. In the previous study of brown rice of 24 Japonica type cultivars in normal and lower temperature cropping years, however, the scatter diagram showed as one group (Taira, 1983b). Consequently, it is presumed that both groups in the scatter diagram are due to the difference between Indica and Japonica types.

Registry No. Palmitic acid, 57-10-3; stearic acid, 57-11-4; arachidic acid, 506-30-9; linoleic acid, 60-33-3; eicosenoic acid, 28933-89-3; lignoceric acid, 557-59-5; oleic acid, 112-80-1.

LITERATURE CITED

Association of Official Analytical Chemists *Official Methods of Analysis*, 12th ed.; AOAC: Washington, DC, 1975; p 497.
Choudhury, N. H.; Juliano, B. O. *Phytochemistry* **1980**, *19*, 1385.
Horiuchi, H.; Tani, T. *Agric. Biol. Chem.* **1966**, *30*, 457.
Hsieh, P. T.; Wu, C. C.; Yang, C. T. *J. Chinese Agric. Chem. Soc.* **1964**, *No. 1, 2*, 1.
Jellum, M. D. *Cereal Chem.* **1970**, *47*, 549.
Jellum, M. D.; Powell, J. B. *Agron. J.* **1971**, *63*, 29.
Juliano, B. O.; Bautista, G. M.; Lugay, J. C.; Reyes, A. C. *J. Agric. Food Chem.* **1964**, *12*, 131.
Taira, H.; Taira, H.; Maeshige, M. *Jpn. J. Crop Sci.* **1979a**, *48*, 220.
Taira, H.; Taira, H.; Fujii, K. *Jpn. J. Crop Sci.* **1979b**, *48*, 371.
Taira, H.; Taira, H.; Ishihara, M. *Jpn. J. Crop Sci.* **1981**, *50*, 109.
Taira, H.; Hiraiwa, S. *Jpn. J. Crop Sci.* **1982**, *51*, 159.
Taira, H. *Rep. Natl. Food Res. Inst. (Jpn.)* **1983a**, *No. 43*, 54.
Taira, H. *JARQ* **1983b**, *16*, 273.
Taira, H. *J. Agric. Food Chem.* **1984**, *32*, 369.
Taira, H.; Kaneko, K.; Haraki, T.; Yamazaki, N.; Ishimaru, H. *Rep. Natl. Food Res. Inst. (Jpn.)* **1985**, *No. 46*, 87.

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Relationship of Disulfide Bonds to the Maintenance of the Active Secondary Structure of Alfalfa (*Medicago sativa*) Leaves Protease Inhibitor

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The polypeptide inhibitor of the alfalfa leaves serine protease contains four disulfide bridges and a high (40–50%) α-helix secondary structure content for an M_r of 6300. The progressive reduction of the four disulfide bridges results in a progressive loss of both the α-helix secondary structure content and the biological activity.

Most of the plant polypeptides with antiproteolytic activity have two common structural peculiarities: i.e. a random-coil structure and a high content of disulfide bridges. Furthermore, all the so far described leaf inhibitor polypeptides show activity only toward nonplant proteases (Ryan, 1981a).

We have recently described (Gonnelli et al., 1985) in alfalfa leaves a novel type of polypeptide showing high specificity and efficiency toward a neutral protease purified from the same source, giving 100% inhibition when the enzyme active site/polypeptide molar ratio is 1. It has four disulfide bonds per M_r of 6300 and an organized secondary structure with a α-helix content ranging between 40 and 50%.

Even though the reduction of disulfide bonds renders the plant polypeptides more susceptible to both thermal denaturation and proteolytic degradation by plant sulfhydryl enzymes they have no particular relevance on the mechanism of inhibition. Plunkett and Ryan (1980) have shown that the potato inhibitor I fully retains its original inhibitory activity after reduction and alkylation of the single monomeric disulfide bond. It has been postulated (Plunkett and Ryan, 1980) that the combined action of S–S bond reduction and the subsequent proteolysis could be important in the in vivo regulation of plant proteolytic activity.

The high content of α-helix secondary structure is a particular feature of alfalfa protease inhibitor polypeptide. To our knowledge (Laskowski and Sealock, 1971) only pancreatic trypsin inhibitor shows some α-helix secondary structure.

Here we report that the progressive reduction of the four disulfide cross-links of the alfalfa protease inhibitor polypeptide brings out a parallel loss of α-helix secondary structure and that this fact per se causes the loss of biological activity.

EXPERIMENTAL SECTION

Materials. The alfalfa leaves protease was purified according to Tozzi et al. (1981); its specific leaf polypeptide inhibitor was purified according to Gonnelli et al. (1985). All the other chemicals were of the highest commercially available quality.

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Preparation of Partially and Fully Reduced Polypeptide Samples. The disulfide bonds of the inhibitor were reduced by incubation (1 mg/mL) for 1, 2, and 15 h at room temperature in 7.5 mM dithiothreitol, 0.05 M Tris-HCl buffer, pH 8.5, previously flushed with, and maintained under, nitrogen. At the indicated times samples were withdrawn and the reduction was terminated by passing the samples through a Sephadex G-10 column (1.5 × 80 cm) equilibrated with 5% acetic acid. The absorbance of 1.5-mL fractions at 280 nm was recorded as they eluted. The fractions containing the polypeptide were collected, lyophilized, dissolved in water, and relyophilized. This procedure was repeated several times to remove the acetic acid. The resulting material dissolved in 10 mM Tris-HCl buffer, pH 8, was used for free sulfhydryl groups titration by the method of Ellman (1959), for the assay of biological activity and for the CD spectral determination.

In our hands the four disulfide bonds show different accessibility to the reducing reagent: under the above reported experimental conditions the first disulfide bridge reacts very quickly (less than 1 h); the second and the third are contemporarily reduced in less than 2 h time, and the last one is cleaved after longer time (over 10 h). Any attempt to prepare samples of polypeptide containing two reduced disulfide bridges by varying either reaction time or dithiothreitol concentration was unsuccessful. Thus, our experiments are limited to polypeptide samples containing none, one, three, and four reduced disulfide bonds.

Protein Determination. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as reference.

Active-Site Determination. The active-site concentration of the alfalfa protease was measured according to the method of Chase and Shaw (1970), using *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride (*p*-NPGB) as the active-site serine titrator.

Protease and Protease Inhibitor Assays. The alfalfa leaf protease was assayed by a spectrophotometric method carried out at 37 °C according to Erlanger et al. (1961). The assay mixture contained 0.01 M Tris-HCl buffer, pH 8, and a 5×10^{-6} M active-site concentration of protease. The reaction was started by addition of N^{α}-benzoyl-DL-arginine-*p*-nitroanilide (DL-BAPNA) solution to a final concentration of 0.23 mM. The final volume was 1 mL. The increase of the optical density at 405 nm was recorded. The inhibitory activity was measured in the above system with 3.8×10^{-6} M inhibitor, which in its native state accounts for 90% inhibition.

CD Spectral Determination. Circular dichroism spectra were measured at room temperature on a Jasco J/500 A spectropolarimeter using inhibitor solutions 4.8×10^{-5} M.

RESULTS AND DISCUSSION

It has been reported (Gonnelli et al., 1985) that in the alfalfa leaf polypeptide inhibitor no free sulfhydryl groups are detectable and that it contains eight half-cystine residues per molecule as measured by amino acid analysis of the acidic hydrolysates. This allowed the hypothesis that all the cysteine residues are present as disulfide bonds. When free SH groups are titred by Ellman method (1959) in samples of polypeptide incubated at 37 °C in the presence of 7.5 mM dithiothreitol for 1, 2, and 15 h, two, six, and eight free SH groups are respectively, revealed. The CD spectra of the native and the reduced polypeptides have been recorded. Figure 1 reports the CD spectra in the 190–240-nm range of the native polypeptide and of the samples partially and fully reduced. The native polypeptide displays the typical CD pattern of the α-helix structure, with the two negative bands at 222 and 208 nm, respectively. The reduction of each of the four bonds results, on the basis of the ellipticity at 222 nm, in a decrease in the α-helix content, the fully reduced polypeptide

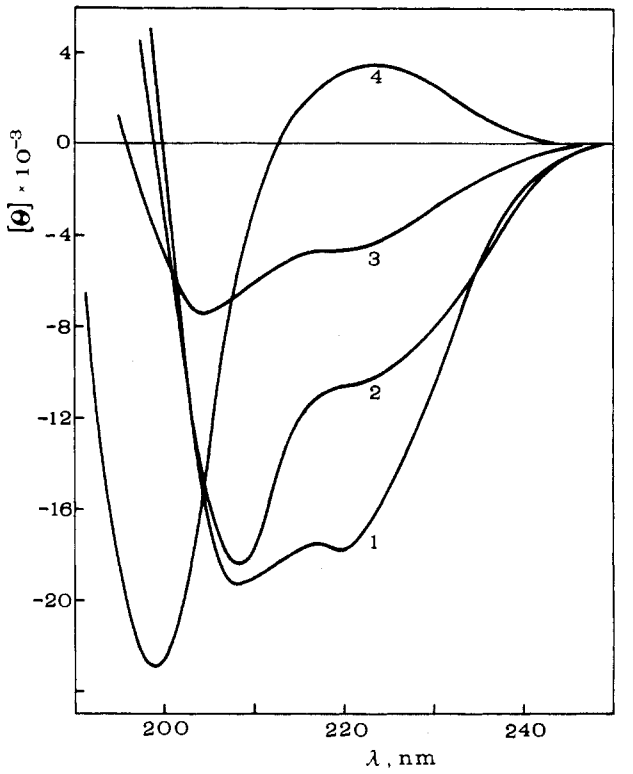

Figure 1. CD spectra of the polypeptide: oxidized form (1); one S–S bridge reduced (2); three S–S bridges reduced (3); fully reduced form (4). Spectra were obtained with 4.8×10^{-5} M inhibitor solutions in 0.01 M Tris-HCl, pH 8.0. Spectra are reported in terms of mean residue ellipticity, [θ].

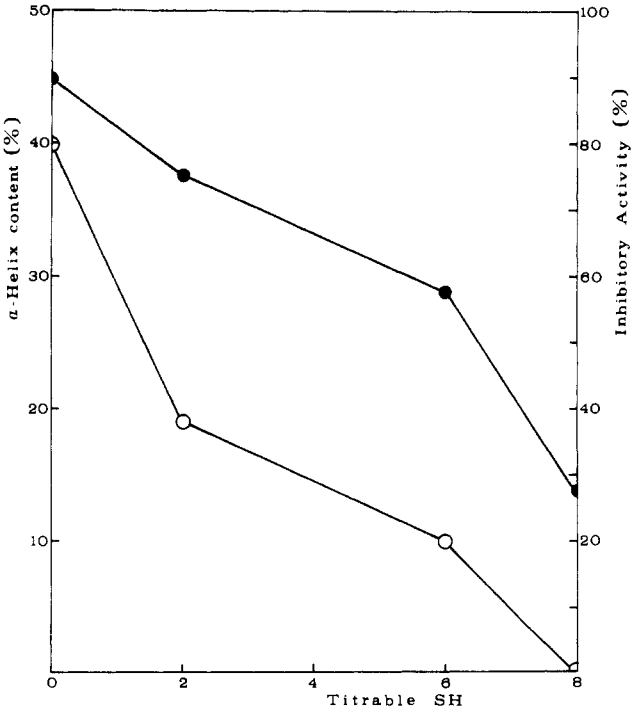

Figure 2. Relationship among percentage of α-helix content (○), inhibitory activity (●), and titrable SH groups. α-Helix content was determined as reported in Figure 1. The assays of the inhibitory activity were carried out at 37 °C in Tris-HCl buffer, pH 8.0, with 3.8×10^{-6} M inhibitor, and the active-site concentration of the inhibited protease was 5.0×10^{-6} M.

showing the CD profile typical of random-coil structures.

Figure 2 reports the relationship between the α-helix secondary structure content and the biological activity of the four tested samples. If the inhibitory activity of the fully oxidized native polypeptide (40% α-helix), is taken as 100%, the sample with 50% of the original α-helix content shows about 85% of residual inhibitory activity. When, following reduction of the second and third bond, the α-helix content falls to 25% of the original, an additional 20% of the biological activity is lost. Finally, the cleavage of the fourth bridge brings up a completely random-coil structure that still maintains a small residual biological activity. Our results indicate that none of the four disulfide bridges is directly involved in the active center of the inhibitor and that their concerted role is to maintain an ordered secondary structure, which is essential for the inhibitory activity of the polypeptide toward its partner protease.

The role of the modulation of the protease activity in the physiology of the plant is well documented (Ryan and Walker-Simmons, 1981b), but there is almost no information concerning the mechanisms of such modulation. Since protease inhibitors are present at every stage of the plant life, one can postulate that the modulation of the activity of the inhibitors could in turn result in the regulation of the protease activity itself. We have recently shown that such modulation in alfalfa leaves may be achieved by varying the endocellular ionic strength (Gonnelli et al., 1982), and here we bring evidence of an additional possible mechanism depending on the α-helix content of the polypeptide.

Registry No. Proteinase inhibitor, 37205-61-1.

LITERATURE CITED

Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248–254.
Chase, T.; Shaw, E. *Methods Enzymol.* **1970**, *19*, 20–27.
Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70–77.
Erlanger, B. F.; Kokowski, N.; Cohen, W. *Arch. Biochem. Biophys.* **1961**, *95*, 271–278.
Gonnelli, M.; Balestreri, E.; Felicioli, R. *J. Agric. Food Chem.* **1982**, *30*, 770–771.
Gonnelli, M.; Cioni, P.; Romagnoli, A.; Gabellieri, E.; Balestreri, E.; Felicioli, R. *Arch. Biochem. Biophys.* **1985**, *238*, 206–212.
Hatch, M. D.; Turner, J. F. *Biochem. J.* **1960**, *76*, 556.
Laskowski, M., Jr.; Sealock, R. W. In *The Enzymes*; Boyer, P., Ed.; Academic: New York, 1971; Vol. 3, pp 375–473.
Plunkett, G.; Ryan, C. A. *J. Biol. Chem.* **1980**, *255*, 2752–2755.
Ryan, C. A. In *The Biochemistry of Plants*; Marcus, A., Ed.; Academic: New York, 1981a; Vol. 6, pp 351–370.
Ryan, C. A.; Walker-Simmons, M. In *The Biochemistry of Plants*; Marcus, A. Ed.; Academic: New York, 1981b; Vol. 6, pp 321–350.
Scalet, M.; Alpi, A.; Picciarelli, P. *J. Plant Physiol.* **1984**, *116*, 133–145.
Tozzi, M. G.; Balestreri, E.; Camici, M.; Felicioli, R.; Ipata, P. L. *J. Agric. Food Chem.* **1981**, *29*, 1075–1078.

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Photosensitized Degradation of a Homogeneous Nonionic Surfactant: Hexaethoxylated 2,6,8-Trimethyl-4-nonanol

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For a better understanding of photochemical interactions between surfactants and herbicides in formulations, a study was conducted with hexaethoxylated 2,6,8-trimethyl-4-nonanol (TMN-6) as a model nonionic surfactant. TMN-6 does not possess chromophoric groups; therefore, photodegradation must take place via the sensitization process. Twelve sensitizing agents ranging in triplet energies from 79 to 29 kcal/mol were examined to obtain an estimated triplet energy of approximately 43–44 kcal/mol for TMN-6. In addition, nine herbicides representing different herbicidal classes were tested as photosensitizers, and six of the nine herbicides sensitized TMN-6 degradation. Solutions were prepared in 30% acetonitrile–water with TMN-6 at 3.3 mM (0.15% w/v) concentration. Products identified from TMN-6 phtodegradation were TMN-5, -4, -3, -2, and -1 and TMNOH (trimethylnonanol) as well as the polyethylene glycols EO-6, EO-5, EO-4, EO-3, EO-2, and EO-1.

Agricultural chemicals of low water solubility are frequently combined with solubilizing agents to yield formulations suitable for field application. Surfactants (cationic, anionic, nonionic) are among those materials used in preparation of agricultural chemical formulations. In some instances as much surfactant, on a weight to weight basis, is applied as active ingredient.

Very few photodegradation experiments have been conducted to investigate the relationship between agricultural chemicals and surfactants. Hautala (1978) has observed an increase in light absorption as well as a small bathochromic shift in the absorption spectrum of 2,4-D and carbaryl with cationic and anionic surfactants. Que Hee et al. (1979) photolyzed the mixed butyl esters of 2,4-D in commercial formulation and found the primary reaction to be reductive dechlorination at the ortho position to yield the butyl esters of (4-chlorophenoxy)acetic acid.

The photochemistry of monuron has been studied extensively (Jordan et al., 1964; Crosby and Tang, 1969; Rosen et al., 1969; Mazzocchi and Rao, 1972; Tanaka et al., 1977); therefore, our initial investigation of surfactant–herbicide interactions was conducted with this compound. The presence of nonionic surfactant in monuron photolysis caused an increase in the photolysis rate, eliminated ring-hydroxylation reactions due to oxidation and substitution, and significantly increased the photoreductive dechlorination reaction (Tanaka et al., 1979). To deter-

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