2) Tertiary butyl alcohol was used as solvent. The reaction product was chromatographed on a column of deactivated alumina. Elution with ether–methanol (50:1) yielded eldelidine (94%).

SUMMARY

The reaction of C_{19}-diterpene alkaloids (eldeline, eldelidine, delcorine, deoxydelcorine, condelphine, lycoctonine, and browniine) with sodium and liquid ammonia in the presence and in the absence of a hydrogen donor – an alcohol – has been studied. It has been established that the transformation of a hydroxy group into a carbonyl group in the absence of a proton donor is characteristic only for C-6-hydroxy or -acetoxy derivatives.

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SPECTRAL-FLUORESCENT PROPERTIES OF MAIZE GRAIN TRYPSIN INHIBITOR

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UDC 535.34+535.372+547.96+633.15

The trypsin inhibitor isolated from maize grain has been investigated by the methods of IR, UV, fluorescence, derivative, and differential spectroscopy. Its spectral characteristics have been determined and the influence of the temperature and of a detergent on the structure of the protein has been studied.

The investigation of spectral characteristics permits the structural features of protein inhibitors of proteinases to be revealed. The nature of absorption in the ultraviolet region of trypsin inhibitors isolated from various plant sources is determined by the ratio of aromatic amino acid residues [1, 2]. Information on the parameters of the UV absorption of a trypsin inhibitor from maize grain has been limited to a determination of its extinction coefficient [3, 4]. Investigations performed by the methods of IR spectrophotometry [5], derivative [6, 7] and differential [7] spectroscopy, and fluorescence [2, 8] are permitting the elucidation of features of the structural organization of plant trypsin inhibitors, but for the inhibitor protein isolated from maize grain the spectral characteristics have not been determined in spite of differences, in comparison with other proteinase inhibitors, in the formation of the secondary structure with a predominance of α-helical sections above the β-structure that have been reported [9].

A comparative investigation of trypsin inhibitors of an initial and a mutant maize that we had carried out previously [10] showed differences in their component compositions.

In view of this, in the present work we have investigated the spectral and fluorescent characteristics of a trypsin inhibitor isolated from the initial maize (line A 204 +++) and that from a maize mutant with respect to the opaque-2 gene (line A 204 o2o2o2).

The IR spectra (cm^{-1}) of the trypsin inhibitor from the grain of the maizes with different genotypes were similar and were typical for proteins:

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Line of maize	Amide A	Amide B	Amide I	Amide II	ν_{C-H}	δ_{C-H}
A 204+++	3304	3070	1656	1540, 1515*	2964, 2935, 2872	1450, 1392
A 204o2o2o2	3300	3080	1658	1536, 1515*	2960, 2935, 2875	1448, 1392

Absorption in the amide I region was represented by a single band, while for amide II one must note the appearance of a shoulder on the main band which may indicate the presence of the different types of regular structures in the protein macromolecule that have been established by the CD method [9, 11].

As is known, the absorption of proteins in the UV region is due to the presence of aromatic amino acid residues. The amino acid sequence of the maize grain trypsin inhibitor includes three tryptophan residues, two tyrosine residues, and one phenylalanine residue [9]. The UV spectra of the trypsin inhibitors from the maizes with different genotypes were similar and were characterized by a maximum at 208 nm having a characteristic inflection at 288.5 nm (Fig. 1). It must be mentioned that the nature of the UV absorption under investigation was close to that of tryptophan, having a high extinction coefficient in comparison with those of tyrosine and phenylalanine [12].

Characteristic for the first-derivative spectrum was the presence of two main negative maxima at 284.5 and 292.5 nm (directed upwards in Fig. 1), a low-intensity negative maximum at 274.5 nm, and positive maxima (directed downwards in Fig. 1) at 277.5 and 288 nm differing in intensity. The second-derivative spectrum revealed the tyrosine and tryptophan components more distinctly, these corresponding to minima at 289.5 and 282.5 nm and maxima at 294, 286, and 276 nm (Fig. 1). The single phenylalanine residue was represented by groups of low-intensity maxima in the 250-270 nm region [13]. Since the values of the maxima in the absorption spectra and also the first and second derivatives of them for the two trypsin inhibitor preparations are close, for a comparative characterization we found the ratio of the optical densities at 260 and 280 nm [14] and also the ratio of the intensity difference 294 nm maximum − 289.5 nm minimum and 286 nm maximum − 282.5 nm minimum, which have been used previously in an investigation of the structural differences of isoenzymes [15] and for determining the amounts of tyrosine and tryptophan residues in proteins [16]. The values obtained for the preparations of trypsin inhibitor from the initial and the mutant maizes did not differ significantly (see below) either in the native state (phosphate buffer, pH 7.7) or in the presence of a detergent ($2 \cdot 10^{-2}$ M solution of sodium dodecyl sulfate, Na-DS). This fact indicated the similar nature of the microenvironments and close ratios of the tyrosine and tryptophan residues in spite of certain difference in the component composition of the preparations isolated from the maizes with different genotypes.

The recording of temperature perturbation differential spectra (TPDSs) made it possible to increase the informativeness of the investigation by studying the temperature changes in the absorption spectra of the proteins due to the interaction of the aromatic chromophores with the environment and not connected with conformational transitions [12]. The TPDS of the trypsin inhibitor obtained at temperatures of 20-35°C (Fig. 2) was determined by the superposition of the tryptophan and tyrosine components and was characterized by positive maxima at 294, 285.5, and 275.5 nm and negative maxima at 288.5 and 279 nm.

Below we give the ratios of the optical densities in the UV spectrum and intensities in the second-derivative spectra of the maize grain trypsin inhibitors.

Solvent	Maize line	$\frac{D_{280}}{D_{260}}$	$\frac{d^2D_{289.5-294}/d\lambda^2}{d^2D_{282.5-286}/d\lambda^2}$
0.1 M phosphate buffer, pH 7.7	A 204 +++	0.68±0.053	1.53±0.227
	A 204o2o2o2	0.70±0.016	1.63±0.027
$2 \cdot 10^{-2}$ M Na-DS in 0.1 M phosphate buffer, pH 7.7	A 204 +++	0.58±0.066	1.38±0.173
	A 204 o2o2o2	0.60±0.087	1.38±0.085

At an excitation wavelength of 208 nm the tryptophan fluorescence predominated in the emission of the trypsin inhibitor, this being characterized by a maximum at 346 nm with a half-width of 58 nm and a quantum yield of 0.11. In comparison with the fluorescence of

*Appears in the form of a shoulder on the main band.

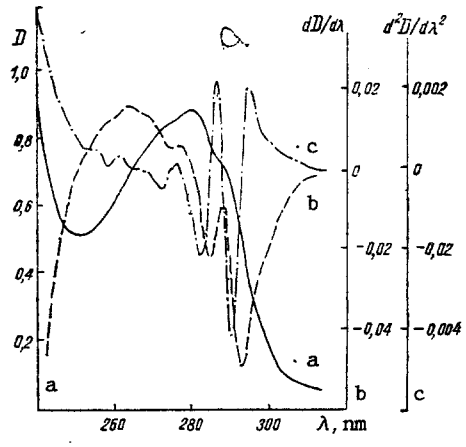

Fig. 1

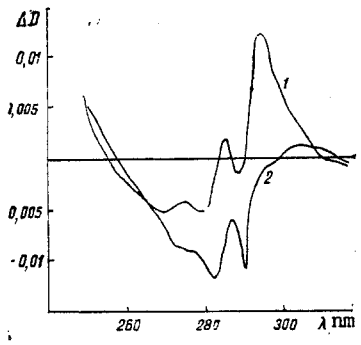

Fig. 2

Fig. 1. UV spectrum (a) and first (b) and second (c) derivative absorption spectra of the maize trypsin inhibitor (line A 204 +++) in 0.1 M phosphate buffer. For curve a the scale of ordinates on the left, and for curves b and c that on the right.

Fig. 2. TPDS of the maize trypsin inhibitor (line A 204 +++) in 0.1 M phosphate buffer (1) and $2 \cdot 10^{-2}$ M Na-DS in 0.1 M phosphate buffer (2); temperature interval 20-35°C.

free tryptophan in aqueous solution, the fluorescence maximum of the protein was shifted in the short-wave direction. The values of the fluorescence maximum and the half-width of the band for the trypsin inhibitor were intermediate for the two structural model forms characterizing the localization of the tryptophan residues on the surface of a compact native or unfolded protein macromolecule [17]. Conversely, the value of the quantum yield coincided with that for the model form with localization of the tryptophanyls in the hydrophobic environment of the protein globule. A similar lack of correspondence of the quantum yield and the position of the fluorescence maximum within the framework of the proposed model has been reported previously for certain proteins and can be explained by the effect of the quenching of the nearest amino acid residues [17].

The characteristics obtained agree with literature information on the fluorescence investigation of trypsin inhibitors from various plant sources. Thus, the trypsin inhibitor from Schizolobium parahybum [2], containing no tryptophan residues, is characterized by tyrosine fluorescence with a maximum at 303 nm and a low quantum yield (0.019). The presence of one tryptophan residue in the trypsin inhibitor from cowpea leads to a marked change in its fluorescence properties: the fluorescence maximum is shifted in the long-wave direction to 355 nm with a sharp rise in the quantum yield (0.12) [8].

The value found for the anisotropy of fluorescence of the trypsin inhibitor (0.089) is low which, with a low viscosity of the microenvironment in dilute solution, may indicate a flexibility of the sections of the polypeptide chain at the positions of binding of the tryptophanyls with their possible localization on the surface of the macromolecule. For comparison, it must be mentioned that the polarization of the fluorescence of the trypsin inhibitor from lima beans, which possesses only tyrosine fluorescence, amounts to 0.12 in aqueous solution [18], which corresponds to a calculated value of the anisotropy of fluorescence of 0.081. At the same time, this value of the anisotropy decreases to 0.055 [18] and is the same for a number of tyrosine-containing proteins on denaturation in 6 M guanidine hydrochloride solution and on the cleavage of the disulfide bonds under the action of β-mercaptoethanol, which confirms the fact that the low values of the anisotropy of fluorescence correspond to accessible fluorophores on the surface of the protein globule.

Earlier, in an investigation of activity and CD spectra [11, 19] it was shown that the structural changes of the maize trypsin inhibitor depend on the nature of the denaturing agents. In view of the sensitivity of derivative spectra to changes in the conformational states of proteins, we have used these methods in an investigation of the trypsin inhibitor after heat treatment and the action of Na-DS.

A solution of the trypsin inhibitor was subjected to heat treatment at 95°C for 10 min since, according to [19], appreciable changes in the magnitude of the activity and the intensity of the CD bands of a protein are observed at temperatures above 80°C. It must be mentioned that the nature of the appearance and position of the bands in the derivative and temperature-perturbation spectra of the trypsin inhibitor after treatment had scarcely changed, which indicates a retention of the microenvironment of the aromatic chromophors.

The action of Na-DS caused more far-reaching conformational changes in the trypsin inhibitor molecule. It had been established previously that at a high concentration of Na-DS ($2 \cdot 10^{-2}$ M) the decrease in the intensity of the CD band did not lead to a loss of inhibitory activity, and this, in the opinion of the authors concerned [19], may be connected with the presence of disulfide bonds which impart conformational stability to the protein in comparison with the maize grain chymotrypsin inhibitor. We have shown that the action of Na-DS ($2 \cdot 10^{-2}$ M) on the trypsin inhibitor molecule is shown in a long-wave shift (1-2 nm) of the positive and negative maxima of the first and second derivative spectra, which is accompanied by a decrease in the relative optical densities in the absorption spectrum and in the intensities in the second-derivative spectrum.

In the presence of the detergent the nature of the TPDS also changed sharply (see Fig. 2): in place of the maximum at 294 nm observed in phosphate buffer, a long-wave maximum was present at 305 nm and an inflection at 295.5 nm. The other TPDS maxima had undergone long-wave shifts to 277, 282.5, 287, and 290 nm. The presence of a TPDS maximum of a number of proteins in the 300-307 nm region is explained by the perturbation of an absorption band, considerably displaced in the long-wave direction, of certain tryptophan residues [12] or by the deprotonation of tyrosine residues at alkaline pH values [20]. The results obtained indicate a change in the nature of the microenvironment of the aromatic chromophores connected with a conformational transition.

Earlier, the TPDS method had shown conformational changes in human serum albumin in the presence of high concentrations of Na-DS [21]. The influence of a detergent on the trypsin inhibitor molecule has a more complex nature than the action of guanidine hydrochloride, leading to the unfolding of the polypeptide chain. It has been shown that Na-DS initiates the formation of α-helices in maize chymotrypsin inhibitor [19] and changes the rigidity of the environment in human serum albumin [21], which can be explained by the binding of the detergent with definite sections of the protein macromolecule [22].

The interaction of the trypsin inhibitor with Na-DS is shown in a long-wave shift of the absorption spectrum revealed by the method of differential spectroscopy. In the differential spectrum of the protein in $2 \cdot 10^{-2}$ M Na-DS solution maxima are observed at 292 and 288 nm (Fig. 3), which is connected with a change in the microenvironment of the tryptophan and tyrosine residues.

EXPERIMENTAL

We used trypsin inhibitor isolated from grain of an initial maize and of a maize mutant with respect to the opaque-2 gene (line A 204) with the aid of affinity chromatography on trypsin-agarose followed by gel filtration on Sephadex G-75, which permitted a 40-fold purification of the preparation with a yield of 40-45% (in terms of activity). The characteristics of the preparations have been given in [10].

IR spectra were investigated on a Specord M 80 spectrophotometer in the 1100-4000 cm^{-1} interval in the form of oriented films which were prepared by depositing 12 ml of an aqueous solution (with a concentration of 2.5 mg/ml) on fluorite glass followed by drying in vacuum.

UV spectra and also first- and second-derivative absorption spectra were obtained on a DU-7 HS instrument in a 1-cm cell at a temperature of 20°C. The concentration of protein in 0.1 M phosphate buffer (pH 7.7) and in a $2 \cdot 10^{-2}$ M solution of Na-DS in phosphate buffer amounted to 0.3 mg/ml. After the addition of the Na-DS the solution was kept at 90°C for 5 min.

The ratio of the optical densities in the absorption spectrum and the intensities in the second derivative spectrum were calculated from four measurements of the spectral characteristics of the preparations from independent isolation experiments.

TPDSs were obtained on a DU-7 HS single-beam spectrophotometer with a microcomputer by a procedure differing from that described for double-beam instruments [22]. The measurement of the TPDS of the trypsin inhibitor was carried out with the use of single cell (1 cm).

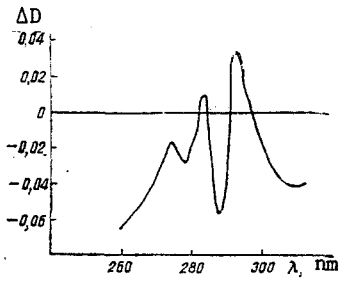

Fig. 3. Differential spectrum of the trypsin inhibitor from maize (of the line A 204 o2o2o2) on the addition of Na-DS to a concentration of $2 \cdot 10^{-2}$ M in 0.1 M phosphate buffer.

The cell with a solution of the protein in 0.1 M phosphate buffer, pH 7.7 (protein concentration 0.6 mg/ml) was thermostated at 20°C for 15 min, and then the absorption of the background was recorded in the scanning regime and the zero line was plotted. Then the temperature was adjusted to 35°C, the solution of the protein was thermostated for 15 min and the TPDS spectrum was recorded, the temperature was again lowered to the initial level (20°C) and, after thermostating, the reproducibility of the zero line was checked. The solution of the protein in the presence of $2 \cdot 10^{-2}$ M Na-DS was kept at 20°C for 30 min before the measurement of the TPDS. The TPDSs were represented in the form recorded by the instrument without allowance for the volume expansion of the solvent [12].

Differential spectra in the 250-320 nm interval were obtained on a Specord M 40 spectrophotometer using paired cells (1 cm) by a published procedure [22]. The initial concentration of protein in the phosphate buffer (pH 7.7) was 0.5 mg/ml. After the zero line had been established, a 10% solution of Na-DS was added to the cell to give a concentration of $2 \cdot 10^{-2}$ M and before recording the spectrum the mixture was kept at 20°C for 30 min.

Heat treatment at 95°C of a solution of the protein for 10 min and thermostating of samples during the spectrophotometric measurement were performed on a DU-7 HS instrument in combination with a device for investigating reaction kinetics and a thermostated sample holder.

Fluorescence spectra were recorded on a MPF-4 spectrofluorimeter in 1-cm cells. The extinction of the protein solution at an excitation wavelength of 280 nm amounted to 0.06-0.08. The quantum yield of the protein was calculated by a comparative method [23]. The quantum yield of tryptophan fluorescence was taken as 0.2 [24]. The fluorescence anistropy r of the trypsin inhibitor was determined on a spectrofluorimeter with a device for investigating polarization and was calculated by means of formula (1) [25]. The recalculation of the polarization P to fluorescence anisotropy for the trypsin inhibitor from lima beans according to the literature [18] was made by means of formula (2) [25]:

$$P = \frac{\bar{I}_{\parallel} - \bar{I}_{\perp}}{I_{\parallel} + 2I_{\perp}} \quad (1)$$

$$r = \frac{2P}{3 - P}, \quad (2)$$

where $I_{\parallel}$ and $I_{\perp}$ are the components of the fluorescence polarized parallel and perpendicular, respectively, to the vector of the vertically polarized exciting light.

To decrease turbidity, the protein solutions were centrifuged at 15,000 rpm for 10 min before the spectrophotometric and fluorescence measurements.

SUMMARY

The parameters of the absorption spectra and of derivatives and temperature-differential spectra are similar for trypsin inhibitors for maizes of different genotypes. The results of fluorescent measurements indicate a possible localization of the tryptophan residues on the surface of the protein macromolecule. According to the results of differential spectroscopy, the trypsin inhibitor is characterized by a high thermal stability, while the action of sodium dodecyl sulfate changed the nature of the microenvironment of the chromophores in the protein.

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