METABOLITES OF THE PATHOGENIC FUNGUS *Verticillium dahliae*. XI. PRIMARY STRUCTURE OF THE POLYPEPTIDE MOIETY OF THE PHYTOTOXIC PIGMENT PKZh-1

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The sequence of the 27 amino acid residues composing the polypeptide moiety of the phytotoxic metabolite PKZh-1 of the fungus *V. dahliae* has been determined on the basis of the structures of tryptic and chymotryptic peptides. It has been established that the linkage of the polypeptide with 2,5,7-trihydroxy-1,4-naphthoquinone (flaviolin), which is the chromophoric moiety of PKZh-1, is effected through an ester bond formed by the carboxy group of the C-terminal amino acid and one of the hydroxy groups of flaviolin.

We have previously given an account of the isolation from the culture liquid of the fungus *V. dahliae*, the causative agent of cotton-plant wilt, of the phytotoxic pigment PKZh-1 and have described its physicochemical properties, the structure of the chromophoric moiety, and its biological activity [1, 2]. In the present paper we give the results of an investigation of the primary structure of the polypeptide moiety of this compound.

PKZh-1 is a strong complex with a trivalent iron ion, which, as has been established, is not precipitated either by alkali or by 8-hydroxyquinoline. To eliminate the Fe^{3+} ions, which interfere with the determination of the amino acid composition of proteins and peptides, we used ion-exchange chromatography, passing a solution of PKZh-1 through a column with the cation-exchange resin Dowex 50W × 2 (H^+ form). To check the binding of the Fe^{3+} ions by the cation-exchange resin, a small part of the substance obtained after the lyophilization of the eluate was subjected to combustion, and the presence of iron ions in the residue was tested by the reaction with potassium hexacyanoferrate(II) and ammonium thiocyanate. Chromatography was repeated until the Fe^{3+} ions had been eliminated completely.

It was found by the dansyl chloride method [3] that the N-terminal amino acid of the polypeptide moiety of PKZh-1 is glycine. The treatment of PKZh-1 with carboxypeptidase A by the method described by Vinogradova [4] did not lead to the splitting off of the C-terminal amino acid. This is possible if the latter is in the form of a C-terminal amide or is one of the basic amino acids, which are not split off by this enzyme [5], or as a consequence of the absence of a free C-terminal amino acid. It was natural to assume that the latter was linked with 2,5,7-trihydroxy-1,4-naphthoquinone (flaviolin), which, as we have established previously [1], is the chromophoric moiety of PKZh-1.

To separate the polypeptide fraction from the flaviolin we used treatment of the PKZh-1, after the iron had been eliminated from it, with a 1 N solution of NaOH. After the reaction mixture had been acidified with dilute hydrochloric acid to pH 3.0, the flaviolin was extracted with ether, and the polypeptide (I) was purified by gel chromatography on Sephadex G-25, using a 0.1 M solution of acetic acid for elution.

After (I) had been treated with carboxypeptidase A, glutamic acid was identified as the C-terminal amino acid. This shows that the linkage of the chromophoric moiety with the polypeptide in the PKZh-1 molecule is effected through an ester bond formed by the carboxy group of the C-terminal amino acid and one of the hydroxy groups of flaviolin.

To determine the molecular weight of (I) we used thin-layer gel chromatography (TLGC) of the carboxymethylated (I) [CM-(I)] on Sephadex G-50 [6]. As the standards we used pro-

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TABLE 1. Amino Acid Composition of Polypeptide (I) and of the Tryptic Peptides

Amino acid	I	Peptide					
		T-1-2	T-2-1	T-3-1	T-4-2	T-5-1	T-6-1
Asp	1.9 (2)	0.9 (1)		1.1 (1)			
Thr	1.8 (2)	0.8 (1)				0.7 (1)	
Ser	1.7 (2)		0.7 (1)			0.8 (1)	0.8 (1)
Glu	3.8 (4)	1.1 (1)		0.9 (1)		1.9 (2)	
Gly	3.9 (4)		2.1 (2)	0.8 (1)	0.8 (1)		2.8 (3)
Ala	0.9 (1)	1.0 (1)					
1/2 Cys	1.7 (2)*					1.8 (2)†	
Val	2.1 (2)			0.9 (1)		0.9 (1)	
Ile	1.1 (1)		0.9 (1)				0.9 (1)
Leu	1.0 (1)				1.0 (1)		1.0 (1)
Tyr	0.9 (1)		0.8 (1)				0.8 (1)
Lys	1.9 (2)			0.9 (1)		0.8 (1)	
His	0.9 (1)					0.9 (1)	
Arg	0.9 (1)				0.8 (1)		0.8 (1)
Met	0.7 (1)		0.7 (1)				0.6 (1)
No. of residues	27	4	6	5	3	9	9
N-Terminal amino acid	Gly	Ala	Ser	Gly	Leu	Cys (SO_3H)	Ser

*Determined in the form of carboxymethylcysteine in hydrolysate of CM-I.
†Determined in the form of cysteic acid.

teins with known molecular weights: adrenocorticotropic hormone (ACTH) (4500), cytochrome c (12,000), and ribonuclease (13,700). On the basis of the linear relationship between mobility and 1 g M of the proteins studied, the molecular weight of (I) was calculated as 3000 ± 100.

The amino acid composition of (I) was determined after acid hydrolysis (Table 1). The presence of tryptophan, which breaks down during the application of this method, was checked in an alkaline hydrolysate of (I) by the qualitative reaction with Ehrlich's reagent and by ion-exchange chromatography on Fixion 50 × 8 plates [5]. In both cases a negative result was obtained.

It was established by Boyer's method of spectrophotometric titration with p-mercuribenzoate (p-MB) [7] that (I) contains no free SH groups. After the reduction of (I) with β-mercaptoethanol, two sulfhydryl groups were detected, which agrees with the results of amino acid analysis. The results obtained show that the two cysteine residues in (I) are linked by a disulfide bond.

To detect and isolate the cystine-containing peptides we used the method of diagonal electrophoresis [8] of the trypic hydrolysate of (I). It was established that the two cysteine residues connected by a disulfide bond were present in one of the tryptic peptides, since only one ninhydrin-positive spot changing its mobility after oxidation with performic acid was detected in the electrophoretogram.

In the determination of the primary structure, to obtain overlapping peptides we used the enzymatic cleavage of (I) by trypsin for 4 h and of CM-I by chymotrypsin for 12 h. The fractionation and purification of the tryptic and chymotryptic peptides was carried out by ion-exchange chromatography on Dowex 50W × 4 cation-exchange resin, by paper chromatography (PC) in systems 1 and 2, and by paper electrophoresis (PE) in pyridine acetate buffer with pH 5.7. The homogeneity of the peptides was judged from the presence of a single spot in each case on PC and PE and also from the N-terminal amino acids, which were determined by Gray's method [3]. The amino acid sequences of all the peptides were determined by Edman degradation in the modification described by Vinogradova [4], with identification of the DNS and PTH derivatives. The latter were used for distinguishing dicarboxylic amino acids from their amides.

On the basis of the amount of lysine and arginine in (I), its treatment with trypsin could be expected to give four peptides. We isolated from a tryptic hydrolysate of (I) six

TABLE 2. Characteristics of the Chymotryptic Peptides

Amino acid	Peptide								
	XT-1-1	XT-2-2	XT-3-1	XT-4-2	XT-5-1*	XT-6-2*	XT-7-1-2	XT-7-2	XT-8-2-1
Asp	0.9 (1)	1,1 (1)							
Thr			0,8 (1)						0,8 (1)
Ser						0,7 (1)	0.8 (1)	0,7 (1)	
Glu	1,1 (1)					1,7 (2)	1,1 (1)	0 8 (1)	
Gly		0,8 (1)		1,8 (2)					0,8 (1)
Ala									1 (1)
Val		1,1 (1)	1,0 (1)					0,9 (1)	
Ile				0,9 (1)					
Leu									1,0 (1)
Tyr				0,8 (1)					
Lys						0,8 (1)	0,8 (1)	0,9 (1)	
His					0,8 (1)				0,7 (1)
Arg									
Met							0,6 (1)	0,6 (1)	
No. of residues	2	3	2	4	2	5	4	5	5
N-terminal amino acid	Asn	Gly	Thr	Gly	Cm Cys	Glu	Glu	Val	Leu

*The peptides each contained one CM-cysteine residue.

peptides, two of which were formed as the result of the cleavage of the tryptic peptides at a tyrosine residue.

As is well known, chymotrypsin possesses a lower specificity than trypsin. On a peptide map of a chymotryptic hydrolysate of a CM-(I) we detected 13-14 ninhydrin-positive compounds, of which the most mobile were the weakest. By this method we isolated in the homogeneous state nine chymotryptic peptides, and one of the ninhydrin-positive substances proved to be free leucine.

Tables 1 and 2 give the amino acid composition of the tryptic and chymotryptic peptides which were used in the establishment of the primary structure of (I). Below we give information on the structures of these peptides.

Peptide T-3-1, Gly-Asp-Val-Glu-Lys, is N-terminal, since in not one of the other tryptic peptides was glycine identified as the N-terminal amino acid.

Peptide T-6-1, Ser-Met-Gly-Ile-Gly-Tyr-Leu-Gly-Arg.

Peptide T-2-1, Ser-Met-Gly-Ile-Gly-Tyr.

Peptide T-4-2, Leu-Gly-Arg.

From a comparative analysis of the amino acid compositions of peptides T-6-1, T-2-1, and T-4-2 and the presence of only one tyrosine and one arginine residue in (I), it follows that the last two peptides are fragments of peptides T-6-1 formed as the result of its cleavage at tyrosine residue. The amino acid sequence of peptide T-6-1 was reconstructed on the basis of the structure of peptides T-2-1 and T-4-2.

Peptide T-1-2, Ala-Thr-Asn-Glu, is C-terminal, since it contains no residues of basic amino acids.

Peptide T-5-1, Cys(SO_3H)-Ser-Gln-Cys(SO_3H)-His-Thr-Val-Glu-Lys.

XT-6-2, Glu-Lys-CmCys-Ser-Gln.

XT-5-1, CmCys-His.

XT-3-1, Thr-Val.

XT-7-2, Val-Glu-Lys-Ser-Met.

The amino acid sequence of peptide T-5-1 was established on the basis of its amino acid composition, the identification of the first six amino acids from the N-end by Edman degradation, and the structure of the chymotryptic peptides given above.

In addition to those mentioned above, the structures of another five chymotryptic peptides were determined:

XT-2-2, Gly-Asp-Val;

XT-1-1, Asn-Glu;

XT-4-2, Gly-Ile-Gly-Tyr;

XT-8-2-1, Leu-Gly-Arg-Ala-Thr;

XT-7-1-2, Glu-Lys-Ser-Met.

The sequence of linkage of the tryptic peptides was determined on the basis of the structures of the chymotryptic peptides containing basic amino acids.

Thus, according to the amino acid sequence of the tryptic and the overlapping chymotryptic peptides, (I) has the following primary structure:

Gly-Asp-Val-Glu-Lys-Cys-Ser-Gln-Cys-His-Thr-Val-Glu-Lys-Ser-Met-
-Gly-Ile-Gly-Tyr-Leu-Gly-Arg-Ala-Thr-Asn-Glu

EXPERIMENTAL

The following solvent systems were used for chromatography: 1) butan-1-ol—pyridine—acetic acid—water (15 : 10 : 3 : 12), 2) butan-1-ol—acetic acid—water (4 : 1 : 5); 3) formic acid—water (1.5 : 100); 4) benzene—acetic acid (9 : 1); 5) n-heptane—n-butanol—acetic acid (3 : 3 : 1); 6) chloroform—methanol (9 : 1); 7) chloroform—ethanol (98 : 2); 8) chloroform—ethanol—methanol (88.2 : 1.8 : 10).

Reduction of the Polypeptide (I). A solution of 40 mg of (I) in 2 ml of a 10 M solution of urea in 0.1 M phosphate buffer (pH 7.5) was treated with 0.1 ml of β-mercaptoethanol. The reaction was carried out in a dark cabinet under nitrogen at 50°C for 1 h. Then the solution was acidified with acetic acid to pH 3.0 and it was deposited on a darkened column (1.5 × 40 cm) containing Sephadex G-25 equilibrated with 0.1 M acetic acid. Elution was carried out with the same solvent, and the fractions containing the reduced (I) were combined, lyophilized, and used to determine the number of free sulfhydryl groups.

The carboxymethylation of (I) was carried out with iodoacetic acid after reduction with β-mercaptoethanol by the method of Crestfield et al [9].

The molecular weight of the CM-(I) was determined by TLGC on Sephadex G-50 Superfine by the method of Belen'kii et al. [6]. A plate with a uniform layer of Sephadex suspension was equilibrated in a closed chamber with a 5 M solution of guanidine chloride for a day. Solutions of carboxymethylated standard proteins and of CM-I were deposited by means of a micropipette, and the course of chromatography was followed from the movement of the brown spot of cytochrome c. A paper replica of the chromatogram was treated with a 0.1% solution of bromphenol blue in methanol—acetic acid (9 : 1). A graph of the dependence of the mobilities of the proteins on log M was plotted, and the molecular weight of the CM-I was calculated from this.

The determination of SH groups in the reduced (I) was carried out by spectrophotometric titration with p-MB [7]. In 0.01-ml portions, a 1 mM solution of p-MB was added to 3 ml of a solution of 0.1 g of reduced (I) in phosphate buffer (pH 7.0). Each successive portion of the p-MB solution was added after the optical density of the solution at 250 nm had ceased to change (about 15-20 min). A graph was plotted of the dependence of the increase in optical density at 250 nm (ΔD_{250}) after the addition of successive portions of p-MB solution on the amount of p-MB consumed. The number of sulfhydryl groups was calculated from the point of inflection on the graph. The titration of the unreduced (I) was not accompanied by a change in the optical density of the solution at 250 nm, which showed the absence of free SH groups.

Cystine-containing peptides were detected and isolated by the method of diagonal electrophoresis of the tryptic hydrolysate of (I). A solution of the hydrolysate was deposited in Filtrak FN No. 17 paper and subjected to electrophoresis in pyridine—acetate buffer (pH 6.5) at a voltage of 1500 V for 1 h. After the end of electrophoresis, a narrow band was cut out from the electrophoretogram and was treated with a solution of ninhydrin to show up the peptides. Guided by this, a second strip 1 cm wide was placed in a desiccator with freshly-prepared performic acid (a mixture of 16 ml of 80% formic acid and 4 ml of 25% hydrogen peroxide), the air was pumped out, and it was left for 2 h. After elimination of the acid vapors in a vacuum desiccator containing NaOH, the dry strip was sewn onto a new sheet of paper and

electrophoretic separation was carried out under the given conditions in a direction perpendicular to the original one. The electrophoretogram after treatment with ninhydrin showed a single spot not located on the diagonal and strongly shifted in the direction of the anode. The method was used for the preparative isolation of this peptide.

Hydrolysis of (I) by Trypsin. Trypsin (Spofa) recrystallized three times from ammonium sulfate [10] and treated with 0.01 N HCl for 16 h to suppress chymotryptic activity was used.

A solution of 100 mg of (I) in 20 ml of 0.1 M ammonium bicarbonate solution was treated with 2 mg of trypsin, and the mixture was incubated at 37°C for 2 h, the pH being maintained with a solution of NH_4OH. Then another 2 mg of trypsin was added and the mixture was incubated for 2 h, after which the solution was acidified with 50% CH_3COOH and lyophilized.

The preparative separation of the peptides from the tryptic hydrolysate was carried out by ion-exchange chromatography in a column (2 × 100 cm) of Dowex 50W × 4 cation-exchange resin (200-400 mesh) at a rate of elution of 60 ml/h, 15-ml fractions being collected. Treatment of the resin, the filling and stabilization of the column, and the analysis of the eluate were carried out as described by Ovchinnikov et al. [11]. For separation we used gradient elution: 2.1 liters of starting 0.2 M pyridine-acetate buffer (pH 3.1), and then a linear gradient obtained by the addition to the mixer containing the starting buffer of 2 M pyridine acetate buffer with pH 5.0 (2.1 liters each) at 40°C, after which 1.5 liters of 2 M buffer was added to the column. Fractions were combined on the basis of the elution curve and were evaporated in vacuum, and the residues were subjected to further purification.

Production and Separation of the Chymotryptic Peptides. A solution of 100 mg of CM-(I) in 20 ml of 0.2 M ammonium bicarbonate was treated with 3 mg of recrystallized chymotrypsin (Serva) and the mixture was slowly stirred at 37°C for 6 h. Then another 3 mg of enzyme was added and incubation was carried out for another 6 h. Hydrolysis was stopped by the addition of acetic acid to pH 3.0, and the reaction products were lyophilized and subjected to fractionation.

The chymotryptic peptides were separated in a column (2 × 120 cm) containing Dowex 50W × 4 cation-exchange resin. The preparation of the column for use was carried out in the same way as in the separation of the tryptic peptides. Fractions with a volume of 15 ml were collected at a rate of elution of 45 ml/h. Elution was begun with the starting 0.2 M pyridine acetate buffer, pH 3.1 (1.2 liters), and then a first gradient was used by passing 0.5 M pyridine acetate buffer with pH 5.0 (2.25 liters) through the mixer containing the starting buffer (2.25 liters), and a second gradient was obtained by feeding to the mixer container 0.5 M buffer a 2 M pyridine acetate buffer with pH 5.0 (2.25 liters each) at a temperature of 50°C. After the end of the gradient, the column was washed with 1.5 liters of 2 M pyridine. The combined fractions were evaporated in vacuum and subjected to further purification.

The tryptic and chymotryptic peptides were purified by chromatography on Filtrak FN No. 17 paper in systems 1 and 2 and by electrophoresis on paper of the same type in pyridine-acetate buffer (pH 5.7) at a voltage of 1000-1500 V for 2-4 h. To detect the peptides, narrow strips of the chromatograms or electrophoretograms were sprayed with a 0.5% solution of ninhydrin in acetone.

The amino acid compositions of (I) and of the tryptic and chymotryptic peptides were determined after acid hydrolysis (5.7 N HCl, 110°C, 24 h) on a Beckman model 119 amino acid analyzer.

The alkaline hydrolysis of (I) was carried out with 2 N NaOH in nitrogen-filled sealed tubes at 105°C for 5 h. The presence of tryptophan in the hydrolysate was checked by the reaction with p-dimethylaminobenzaldehyde and by ion-exchange chromatography with a marker on Fixion 50 × 8 plates (Chinoin), as described by Devenyi and Gergely [5].

N-Terminal acids were determined by Gray's method. The DNS derivatives of the amino acids were identified by two-dimensional chromatography on plates (6 × 6 cm) with a fixed layer of type KSK silica gel in the systems given by Vinogradova et al. [4] and on plates coated with polyamide in system 3 in one direction and in system 4 in the perpendicular direction. System 5 was used for the identification of the DNS derivatives of the basic amino acids and threonine and serine. The polyamide plates were prepared as described by Reshetov [12].

Determination of the C-terminal Amino Acid in (I). A mixture of 0.04 ml of a suspension of carboxypeptidase A with 3 ml of cooled 0.1 M NaCl solution was stirred and 0.05 M NaOH

solution was added until the enzyme had dissolved completely (pH 10), its concentration then being determined spectrophotometrically at 280 nm. A solution of 0.3 mg of (I) in 0.5 ml of 0.05 M triethylamine carbonate buffer (pH 7.8) was treated with a solution of carboxypeptidase A (at an enzyme : substrate ratio of 1 : 50), and the mixture was incubated at 30°C. Samples (0.1 ml) were taken after predetermined intervals of time (5, 15, 30, 60, and 240 min). The reaction was stopped by acidification with acetic acid to pH 1-2. Then the mixture was freeze-dried and its amino acid content was determined.

The amino acid sequences of the peptides were determined by Edman degradation in the modification described by Vinogradova et al. [4] with identification of the amino acids in the form of the PTH and DNS derivatives. The latter were identified as described above, and the PTH derivatives by one-dimensional chromatography on plates (6 × 6 cm) coated with type LS_{254} silica (5/40 μ + 13% of gypsum) from Chemapol in systems 6, 7, and 8, being revealed in the form of dark spots when the plates was illuminated with UV light.

SUMMARY

The primary structure of the polypeptide moiety of the phytotoxic metabolite PKZh-1 from the fungus *V. dahliae* the causative agent of cotton-plant wilt, has been determined.

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