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CERATO-ULMIN—A WILTING TOXIN OF DUTCH ELM DISEASE FUNGUS

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Key Word Index—*Ceratocystis ulmi*; Dutch elm disease; cerato-ulmin; wilting toxin; protein, MW; amino acid composition; amino acid sequence.

Abstract—Cerato-ulmin, a toxin produced by *Ceratocystis ulmi*, the causal agent of Dutch elm disease, has been characterized as a small protein (128 residues) with a MW of *ca* 13000. The protein has a high content of cystine, proline, leucine, serine and aspartic acid/asparagine; it is low in histidine, lysine, arginine, isoleucine, phenylalanine and tyrosine and does not contain cysteine, methionine, or tryptophan. The amino acid sequence of the *N*-terminal region is: H_2N-Ala-Asp-Ser-Tyr-Asp-Pro-Cys-Thr-Gly-Leu-Leu-Gln-Lys-Ser-Pro-Gln-Cys-Cys-Asp-Thr-Asp-Ile-Leu-Gly-Val-Ser-Asp-Leu-Asp-Cys-. Toxic symptoms similar to those of Dutch elm disease can be elicited by cerato-ulmin in white elm shoot cuttings (*Ulmus americana* L.).

INTRODUCTION

Dutch elm disease is caused by the fungus *Ceratocystis ulmi* (Buism.) C. Moreau. Although toxins produced by this fungus were mainly associated with large polysaccharides (MW 0.5–2.0 × 10^6) [1–8], characterization has been incomplete. In contrast to these findings, studies by Takai [9], Richards and Takai [10] and Takai and Richards [11] led to the isolation of a toxic substance excreted by *C. ulmi*, that is a protein with a low carbohydrate content. This substance has lyotropic liquid crystal-like properties that allow it to associate in aqueous media to yield polymer-like microstructures termed 'fibrils' [9–11]. These structures impart a milky appearance to an aqueous solution. Transformation of 'fibrils' into 'units' or 'monomers' occurs when the solution is subjected to centrifugation, pressure, chilling (~0°) or, alternatively, when the solution is made 60 to 80% with respect to ethanol or 8M in urea [11]. The transformation occurring in ethanol or urea suggests that hydrophobic interactions occur between monomers. An interesting property of this toxin is its solubility in high concentrations of ethanol. This characteristic has greatly assisted in its isolation and purification [10, 11].

Liquid shake cultures of aggressive strains of *C. ulmi* grown in defined media readily produced substantial quantities of toxin (*ca* 140 mg/l. of culture filtrate) [9, 10, 12]. The toxin has been isolated free of other proteins and has been named cerato-ulmin [9, 11]. The administration of cerato-ulmin to white elm cuttings (*Ulmus americana* L.) causes wilting, necrosis and chlorosis that are similar to symptoms of Dutch elm disease [9, 11]. The lower range of effectiveness of cerato-ulmin that is capable of producing the Dutch elm disease symptoms in cuttings is 2 ng/ml [13]. Cerato-ulmin has the general features of a pathotoxin as defined by Wheeler and Luke [14].

This paper reports studies on the characterization of cerato-ulmin with respect to its MW, amino acid composition and partial amino acid sequence.

RESULTS

Cerato-ulmin was purified as outlined by Takai and Richards [11] except that the final step was gel filtration on Sephadex LH60 instead of Sephadex LH20. Cerato-ulmin was excluded from Sephadex LH20 and was eluted in the breakthrough peak [11]. Using Sephadex LH60 gel filtration in 60% ethanol, cerato-ulmin entered the gel and was resolved (peak A) from two contaminants (peak B and C). The material under each peak was pooled and diluted 10-fold with water and freeze-dried. Only the material obtained from peak A yielded a fluffy white substance commonly observed with proteins. Moreover, only the aqueous solution of peak A possessed a milky appearance, indicative of polymer formation, routinely observed with cerato-ulmin. Amino acid analyses (after acid hydrolysis) of peaks A, B and C and the very minor peaks preceding and following these major peaks, revealed that only peak A was a polypeptide since the others yielded traces of amino acids by comparison. The high absorbance (280 nm) of peaks B and C is ascribed to the presence of a phenolic pigment. Amino acid compositions of cerato-ulmin obtained from either Sephadex LH20 [11] or LH60 gel filtration were identical, indicating that the same polypeptide was present in both preparations.

Polyacrylamide gel electrophoresis in 1% SDS indicated that cerato-ulmin, obtained from gel filtration on Sephadex LH-60, was not contaminated with other proteins. Free carbohydrates could be present but would not be detected by staining with Coomassie Brilliant Blue. Increasing the concentration of cerato-ulmin on the gel 15-fold did not reveal minor contaminating proteins.

A plot of the log MW vs mobility of pepsin, chymo-

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trypsinogen A, myoglobin, lysozyme, ribonuclease A, and cerato-ulmin indicated that the MW of the cerato-ulmin was 13370 ± 770. Gel electrophoresis using BDH low range MW markers and plotting the data as above suggested a MW of 13000 ± 600. The BDH markers were comprised of monomer, dimer, trimer to oligomers of a single protein and the data obtained showed a linear relationship. This estimate of the MW of cerato-ulmin was, therefore, considered more reliable.

The high speed sedimentation equilibrium centrifugation of procedures of Yphantis [15] indicated a MW of 8480 which was considerably lower than the values determined by polyacrylamide gel electrophoresis and amino acid analysis (12726; *cf.* Table 1). However a value for the partial specific volume was assumed to be 0.730 g/ml and has not been determined for cerato-ulmin. A linear graph obtained from a plot of the log of the fringe displacement vs r^2 over the entire cell indicated that cerato-ulmin was homogeneous.

Amino acid analyses of cerato-ulmin is presented in Table 1. The native protein was subjected to acid hydrolysis in 6 M HCl at 110° for 24, 48 and 72 hr. Triplicate analyses were performed with average recoveries calculated for all amino acids except for serine/threonine and valine/isoleucine where extrapolation to zero time or 100 hr, respectively, were performed. The content of half-cystine was calculated as 12.5 residues but a more accurate determination, as *S*-carboxymethyl cysteine (14 residues), was obtained by reduction and carboxyamidomethylation.

Cerato-ulmin does not possess free sulfhydryl groups.

Table 1. Amino acid composition of cerato-ulmin

Amino acid Hydrolysis Time	Amino acid composition 24 hr	48 hr	72 hr	Average value	Integral value
Lys	1.38	1.32	1.30	1.33	1
His	1.22	1.20	1.23	1.22	1
Arg	3.04	2.96	2.87	2.84	3
Asp	14.12	13.91	13.86	13.96	14
Thr	8.71	8.17	7.52	9.35*	9
Ser	15.15	14.42	13.15	16.30*	16
Glu	6.90	6.44	6.53	6.62	7
Pro	11.52	11.39	12.46	11.79	12
Gly	12.31	11.99	11.59	11.96	12
Ala	8.60	8.45	8.16	8.40	8
Half-cys (*S*-Carboxymethyl cysteine)	11.86 (14.35)	12.76	12.70	12.44	12–14 14
Val	7.63	8.23	8.37	8.81†	9
Met	0	0	0	0	0
Ile	2.84	2.97	2.98	3.02†	3
Leu	16.00	16.00	16.00	16.00	16
Tyr	1.55	1.07	0.91	1.18	1
Phe	1.57	1.45	1.55	1.51	2
Trp‡	0	0	0	0	0
				Total:	128
				Minimum MW:	12933

The value for leucine is arbitrarily set at 16.0 residues, for an average of three analyses per hydrolysis time.

* Extrapolated to zero time.

† Extrapolated to 100 hr.

‡ Based on recovery to tryptophan after hydrolysis in 4 M methane sulphonic acid containing 0.2% tryptamine [16] and also on Ehrlich's reagent test for tryptophan (Bailey, [17]).

Under alkaline denaturing conditions (as used in reduction and alkylation section of Experimental but, in this case, not containing Bu_3P), treatment of cerato-ulmin with iodoacetamide did not yield *S*-carboxymethyl cysteine following acid hydrolysis and amino acid analysis.

Tryptophan was not present in cerato-ulmin as indicated by amino acid analysis following hydrolysis in 4 M methane sulfonic acid containing 0.2% tryptamine [16]. Under these conditons, chymotrypsinogen A yielded the expected 8 residues of tryptophan. Proteinase digests of cerato-ulmin applied to Whatman 3 MM paper did not yield a positive violet colour with *p*-dimethylaminobenzaldehyde (Ehrlich's reagent) thereby further indicating that tryptophan was absent [17].

Methionine was also not present in cerato-ulmin. The values shown for aspartic and glutamic acids represent the sum of asparagine/aspartic acid and glutamine/glutamic acid, respectively, that are present in cerato-ulmin. All ninhydrin positive compounds observed on the amino acid chromatogram have been identified.

Based on the amino acid composition of cerato-ulmin (Table 1) and setting the value of leucine equal to 16 residues, the protein contains 128 residues and has a MW of 12933. The MW is in agreement with estimates from polyacrylamide gel electrophoresis.

Cerato-ulmin isolated by Takai and Richards [11] contained 10.3% carbohydrate. New determinations on cerato-ulmin twice chromatographed on Sephadex LH20 yielded values of 5.6% ± 0.2. The carbohydrate content of cerato-ulmin obtained from Sephadex LH60 (peak A) was 4.4% ± 0.2. Amino acid analyses of the acid hydrolysate of cerato-ulmin did not reveal the presence of the common amino sugars glucosamine, galactosamine and mannosamine. These compounds, if present, would have been detected (*cf.* Experimental).

A partial amino acid sequence of the *N*-terminal region of cerato-ulmin is presented in Fig. 1. The identification of each residue was obtained as outlined in Experimental. The sequence represents *ca* 25% of the entire protein.

The toxicity of cerato-ulmin isolated by procedures presented in this study was identical to that obtained for cerato-ulmin isolated by Takai and Richards [11]. Toxic symptoms were elicited by cerato-ulmin in white elm shoot cuttings at a concentration of 10 μg/ml. However, cerato-ulmin can elicit toxic symptoms in concentrations as low as 2 ng/ml [13], implicating this protein in the etiology of Dutch elm disease. Reduction and carboxymethylation of cerato-ulmin destroyed the native tertiary structure while maintaining the single polypeptide chain. This form of cerato-ulmin was inactive in the bioassay.

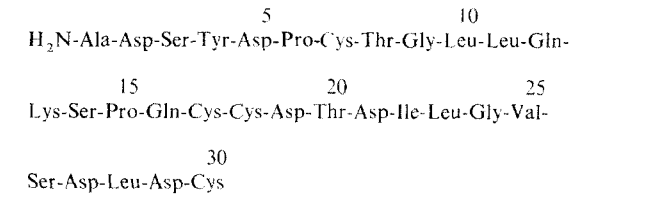

Fig. 1. The amino acid sequence of the *N*-terminal region of cerato-ulmin—a toxin produced by *C. ulmi* the fungus responsible for the Dutch elm disease. Cys refers to cysteine that was present as the *S*-carboxyamidomethyl-[^{14}C] cysteine derivative. In the native protein, Cys residues are in the form of disulfide bridges.

DISCUSSION

Cerato-ulmin produced by the fungus *Ceratoycystis ulmi*, the causal agent of Dutch elm disease, has been characterized as a small protein of 128 residues with MW *ca* 13000. The amino acid composition of cerato-ulmin is unusual with the high amounts of proline (12 residues) and 7 disulfide bridges. These residues, together with the high content of serine (16 residues), threonine (9 residues) and valine (9 residues), which are known to be helix breakers [27, 28], suggests that cerato-ulmin could possess a compact globular structure. The large amount (~30%) of hydrophobic residues (Ala, 8; Val, 9; Ile, 3; Leu, 16; Tyr, 1; Phe, 2) are responsible, in part, for the peculiar solubility properties of cerato-ulmin in 60–80% ethanol. The tendency of the toxin to polymerize in aqueous solution [11] and to dissociate in largely nonpolar solutions is suggestive of extensive hydrophobic interactions.

Cerato-ulmin is probably not a glycoprotein. New isolation procedures using gel filtration on Sephadex LH60 in 60% ethanol yielded cerato-ulmin with a carbohydrate content of 4.4%. This is considered to be a contaminant of free carbohydrate and not covalently bound to the protein. No evidence for amino sugars was observed on amino acid analyses and the small amount of carbohydrate actually present probably arises from the incomplete separation of a carbohydrate contaminant.

The amino acid sequence of the *N*-terminal region (Fig. 1) has no apparent homology with published protein sequences [18, 19]. The location of the single lysine and tyrosine residues has been determined. Studies on the elucidation of the complete amino acid sequence and the order of disulfide bridges are in progress.

Several toxic glycopeptides have been isolated from *C. ulmi* cultures (Salemink *et al.* [6], Rebel [7], van Alfen and Turner [8] and Strobel *et al.* [29]). Due to a lack of clear descriptions, it is difficult to determine whether or not the same toxins were studied by these laboratories. These toxins, however, are quite different from cerato-ulmin in the following respects. Cerato-ulmin has a MW of *ca* 13000 whereas the MWs of the glycopeptide toxins are much greater (2.5×10^4 and 10^6 [6]; 3×10^4 and 10^6 [7]; 5×10^5 and 2×10^6 [8]; 7×10^4 and 2.7×10^5 [29]). The preparation of cerato-ulmin contains 95.6% protein and only 4.4% carbohydrate. The polypeptide exists as a single chain of which 30 residues have been sequenced. In marked contrast, the toxins studied in other laboratories have the opposite compositions (90% carbohydrate [7]; 95% carbohydrate, 5% protein [8]; 83.5% carbohydrate, 6.7% protein [29]). Cerato-ulmin has a well defined amino acid composition compared to other toxins reported [29]. Finally, cerato-ulmin is the only toxin produced by *C. ulmi* to possess liquid crystalline characteristics [9–11].

EXPERIMENTAL

Materials. Iodoacetamide and MW marker (Range: 14300–71500) were purchased from B.D.H. *n*-Tributylphosphine was obtained from Aldrich Chemicals. SDS (sequanal grade) and mercaptoethanol were from Pierce. Chicken egg-white lysozyme and myoglobin were from Sigma. Chymotrypsinogen A and pepsin were from Worthington. Ribonuclease A (pancreatic) was from Nutritional (ICN) Biochemicals. Iodoacetamide [1-^{14}C], 15.76 mCi/mmol, was from New England Nuclear. Spectrapor dialysis membrane tubing (MW cutoff 6000–8000) was purchased from Spectrum Medical Industries, Inc., Los Angeles, California.

Growth of Ceratocystis ulmi *isolate CESS 16K* (American Type culture collection Code: ATCC 34359) was carried out as described in refs. [11, 12].

Isolation of cerato-ulmin was as outlined in ref. [11]. The toxin (40 mg) was suspended in 2 ml of 60% EtOH and centrifuged to remove undissolved material. The supernatant was applied to a 1.6 × 25 cm Sephadex LH60 column equilibrated and eluted with 60% EtOH at 23°. The flow rate was 10 ml/hr with 1 ml fractions being collected. The elution profile was monitored at 280 nm. The fractions, containing material absorbing at 280 nm, were suitably pooled, diluted 10-fold with H_2O and freeze-dried. The recovery of pure cerato-ulmin was *ca* 10 mg.

Ultracentrifugation analyses were performed by the high-speed methods of ref. [15]. The concn of cerato-ulmin was 0.5 mg/ml and the protein was dialysed for 12 hr against 3 changes of 0.1 M KPi, pH 7. The runs were performed at 52000 rpm for a period of 28 hr.

Polyacrylamide gel electrophoresis was carried out as outlined in ref. [21]. Gels consisting of 10% T(w/c) and 2.6% C (bisacrylamide per total acrylamide) were buffered with 0.1 M NaPi buffer, pH 6.7, containing 1% SDS. Proteins were prepared in solns containing 0.01 M NaPi, pH 6.7, 1% glycerol, 1% SDS and 0.1% mercaptoethanol at concns of 1.5 mg/ml (chymotrypsinogen A, ribonuclease A, lysozyme, pepsin, cerato-ulmin) or 4.5 mg/ml (myoglobin). These protein solns were heated at 100° for 1 min. Aliquots (10 µl) were applied to a slab gel (10 × 10 cm) and were run at 110 mA for 3.5 hr. The slab gel was fixed with 25% TCA for 30 min and stained with Coomassie Brilliant Blue (0.25% in 25% TCA) for 1 hr at 37°. Destaining was carried out electrophoretically in a Pharmacia gel destainer. Estimates of the MW of cerato-ulmin were obtained from plots of the log MWs vs mobility of standards during electrophoresis.

Cerato-ulmin concentration was determined in 60% EtOH or in solns containing 8 M urea by measuring the A at 280 nm and using the relationship $E^{1\%}_{1cm} = 2.0$.

Reduction and alkylation of cerato-ulmin was performed by a modification of the method of ref. [22]. Cerato-ulmin (5 mg, 5×10^{-4} mmol) was added to a soln containing 0.7 ml of 0.2 M $NaHCO_3$, 0.7 ml of *iso*PrOH and 1 g of urea. Final vol. was 2 ml. Iodoacetamide (1.85 mg, 10^{-2} mmol) was introduced in 50 µl of *iso*PrOH and the reduction of disulfide bridges was initiated by the addition of *n*-tributylphosphine (Bu_3P) (15 µl, 6×10^{-2} mmol). The soln was maintained at 37° for 3 hr. The alkylated cerato-ulmin was isolated from reactants and urea by gel filtration on Sephadex G-25 (1.2 × 50 cm) or Sephadex G-15 (2.5 × 43 cm) equilibrated with 10 mM HOAc and was freeze-dried. Alternatively, diluting the reduction soln 10-fold with H_2O and dialysing 24 hr against 4 changes of H_2O (6 l.) was employed prior to freeze-drying.

Cerato-ulmin labelled with iodoacetamide-[^{14}C] was carried out at noted above but using initially 0.3 mg of iodoacetamide-[1-^{14}C] (25 µCi) in 250 µl of EtOH. After 1 hr the procedure was continued, as noted above, using unlabelled iodoacetamide. The reduction method using Bu_3P was verified with pancreatic ribonuclease A. Quantitative alkylation of the reduced ribonuclease was obtained in this procedure using iodoacetamide, iodoacetic acid, ethylenimine or propane sultone (K. J. Stevenson, unpublished results [23]).

Amino acid analyses were performed on a Beckman 121 automatic amino acid analyser by the method ref. [24] as modified for single column analyses. The buffers employed were 0.2 N Na citrate pH 3.25, 0.4 N Na citrate, pH 4.10 and 1 N Na citrate pH 6.5 supplied as concentrates by Beckman Instruments Inc., Palo Alto, California. Triplicate analyses were carried out on cerato-ulmin hydrolysed in 6 M HCl at 110° for 24, 48 and 72 hr. Half-cysteine was determined as *S*-carboxymethyl cysteine with appropriate corrections being employed [25]. The use of the pH 3.25 Na citrate buffer (in preference to pH 3.49) enabled *S*-carboxymethyl cysteine to be eluted well in advance of

aspartic acid. Common amino sugars were readily detected in this system since glucosamine eluted just ahead of tyrosine and galactosamine and mannosamine eluted between tyrosine and phenylalanine. Samples for tryptophan analyses were hydrolysed in 4 M methanesulphonic acid, containing 0.2% 3-(2-aminoethyl)-indole for 24 hr at 110° [16].

Amino acid sequence analyses were performed with carboxyamidomethyl-[^{14}C] cerato-ulmin using a Beckman Model 890C Sequencer employing 0.5 M dimethylallylamine in 50% PrOH adjusted to pH 9.85 with trifluoracetic acid. A portion of the thiazoline derivatives was converted to the corresponding amino acids using back-hydrolysis in 47% HI under N_2 at 130° for 20 hr [26]. Amino acid analyses were performed on a Beckman 121 M amino acid microanalyser. The position of *S*-carboxyamidomethyl cysteine in the amino acid sequence was identified by the presence of the ^{14}C-label as revealed by liquid scintillation counting. Subjecting the acid hydrolysate of carboxyamidomethyl-[^{14}C] cerato-ulmin to qualitative amino acid analysis on high voltage electrophoresis at pH 2 [17, 23] indicated that the ^{14}C-label was only associated with *S*-carboxymethyl cysteine.

Bioassay of toxicity. Cerato-ulmin was dissolved in H_2O (10 mg/ml) and centrifuged at 1465 *g* for 30 min to clear the soln thus ensuring that cerato-ulmin was in the monomer form [9, 11]. An aliquot of this soln was added to a test tube containing a cutting of white elm shoot (*Ulmus americana* L.) in H_2O. Final concn of cerato-ulmin was 10 μg/ml. The tube was sealed with Parafilm and place in a controlled growth chamber (50% relative humidity, 25° 6.4 klx of light). Care was taken not to cause air locks in the conducting systems of the elm shoot during cutting and transfer into H_2O. Periodically, cuttings were observed for external symptom expression (drooping and wilting) and measurements of transpiration were obtained as wt differences between readings of fr. wt of the cutting taken during the test period.

Carbohydrate determination. The carbohydrate determinations was performed using the PhOH–H_2SO_4 method [20].

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