Volume 257, number 2, 302–306 FEB 07775 November 1989

Amino acid sequence of cinnamomin, a new member of the elicitin family, and its comparison to cryptogein and capsicein

Jean-Claude Huet and Jean-Claude Pernollet

Laboratoire d'Etude des Protéines, Centre INRA, route de St Cyr, 78026 Versailles Cedex, France

Received 5 September 1989

The phytopathogenic fungi *Phytophthora cinnamomi* cause systemic leaf necrosis on its non-host tobacco; in culture, it secretes a protein, called cinnamomin, which elicits leaf necrosis and protects tobacco against the pathogen *Phytophthora nicotianæ*, in a way similar to cryptogein and different from capsicein, elicitins of known amino acid sequences. The cinnamomin sequence has been determined and compared to other elicitins. The differences in the 3 elicitin sequences were correlated to the biological acivities: 2 lysines were ascribed as the key amino acids involved in the differential control of protection with respect to necrosis.

Amino acid sequence; Elicitin; Plant pathogen; (*Phytophthora, Nicotiana tabacum*)

1. INTRODUCTION

Upon inoculation, *Phytophthora nicotianae*, the agent of the tobacco black shank, invades tobacco stems, whereas other *Phytophthora* species cause limited colonization accompanied by leaf necrosis at a distance from the inoculation court. In contrast, no distant leaf necrosis is observed during the interaction between *P. nicotianae* and tobacco, and no elicitor of necrosis has been detected in culture filtrates [1]. In culture, *P. cryptogea, P. capsici* and *P. cinnamomi* secrete low molecular mass proteins named cryptogein, capsicein and cinnamomin, respectively [2], which have been purified [3]. These fungal proteins constitute a novel protein family, called elicitins. Applied on tobacco plants, elicitins not only elicit leaf necrosis, but also cause the accumulation of pathogenesis-related proteins [4] and induce protection against a subsequent inoculation with the tobacco pathogen *P. nicotianae*. Elicitins from various species exhibit different levels of biological activities, abolished upon pronase digestion [5]: cryptogein causes visible leaf necrosis when applied at ~1 μg per plant, whereas 50-fold as much capsicein is required for the same reaction; in contrast to cryptogein, capsicein induces protection even in the near absence of leaf necrosis. The complete amino acid sequences of these two elicitins are known: they are of similar M_r (respectively 10323 and 10155) and consist of 98 residues. They are holoproteins devoid of glycosylation which exhibit a 85% homology: only 2 short terminal regions, which should be involved in the modulation of the biological activities which distinguish cryptogein from capsicein, are heterologous [5].

Culture filtrates of *P. cinnamomi* induce protection of tobacco against its pathogen [4]. Cinnamomin, purified from these filtrates [3], shows a level of necrotic activity comparable to cryptogein [1,3] and protects tobacco against its pathogen at similar doses. We have therefore determined the amino acid sequence of cinnamomin to get a more precise knowledge of the amino acid residues which are involved in the modulation of the elicitin biological activities.

Correspondence address: J.-C. Pernollet, Laboratoire d'Etude des Protéines, Centre INRA, route de St Cyr, 78026 Versailles Cedex, France

Abbreviations: Asp-N: Asp-N endoproteinase, EC 3.4.21; Cap, capsicein; Cin, cinnamomin; Cry, cryptogein; RPLC, reversed-phase liquid chromatography

2. MATERIALS AND METHODS

2.1. *Material*

Cinnamomin was purified as already described [3] and was reduced with dithiothreitol and *S*-carboxymethylated with unlabeled iodoacetic acid according to Fernandez-Luna et al. [6].

2.2. *CNBr, Asp-N and tryptic digests*

CNBr peptides were obtained as follows: *S*-carboxymethylated protein was dissolved in 70% (v/v) formic acid (20 mg/ml). After addition of a 25-fold weight excess of cyanogen bromide over the methionine content, the mixture was incubated for 24 h at room temperature in the dark; the reaction mixture was then diluted 5-fold with cold distilled water and lyophilized. Asp-N endoproteinase (EC 3.4.21), which allows the cleavage at the amino acid side of Asp and $CysSO_3H$ [7], purchased from Boehringer, was used for the cleavage of 0.2 mg of *S*-carboxymethylated cinnamomin (1 mg/ml) with an enzyme to substrate weight ratio of 1:100, for 18 h at 37°C in 50 mM Na phosphate buffer, pH 8. Tryptic peptides were obtained using *S*-carboxymethylated protein, dissolved in 0.2 M *N*-ethylmorpholine acetate buffer, pH 8.2 (10 mg/ml), and incubated with trypsin (EC

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00145793/89/$3.50

Table 1

Edman amino acid sequencing of cinnamomin

Peptide denomination	N-term	T1	T2	T3	T4	T5	T6	T7	A	B
Elution position (CH_3CN%):	30	9	10	12	21	26	30	32.5	10	25
Cycle										
1	T (1630)	A(1580)	C(2970)	T(520)	L (710)	D (320)	T(350)	I (500)	D*(240)	I (147)
2	A (4300)	N(970)	A(3230)	A(1000)	M(890)	S (160)	L(630)	V(465)	G (170)	K(99)
3	C (2550)	G(670)	S (1740)	C(960)	C (590)	G (140)	V(490)	A(510)	F (180)	K(13)
4	T (3330)	F (630)	L(470)	T(340)	A (630)	Y (160)	S (580)	L(430)	S (28)	I (140)
5	A (3520)	S (560)		A(960)	S (530)	S (130)	I (470)	N(410)	S (10)	V(128)
6	T (2090)	S (-150)		T(310)	T (170)	M(81)	L(440)	P(420)	K (89)	A(140)
7	Q (2570)	K(180)		Q(800)	A (390)	L (80)	S (440)	P(-32)	C (100)	L(128)
8	Q (440)			Q(-17)	C (290)	T (28)	E(350)	D(170)	A (100)	N(134)
9	T (1890)			T(250)	N (290)	A (45)	S (150)	C(200)	S (20)	P(122)
10	A (2350)			A(590)	T (110)	T (25)	S (73)	D(99)	L (8)	P(8)
11	A (610)			A(26)	M(150)	A (28)	F (170)	L (190)		D(78)
12	Y (1810)			Y(390)	I (94)	L (50)	S (21)	T(68)		C(70)
13	K (1760)			K(230)	K (63)	P (42)	Q(130)	V(120)		D(51)
14	T (1180)					T (16)	C(67)	P (100)		L(41)
15	L (1270)					N (29)	S (40)	T(24)		T(33)
16	V (990)					A (20)	K(35)	S (110)		V(37)
17	S (1030)					□		G(51)		P(22)
18	I (810)							L(48)		T(16)
19	L (690)							V(43)		S (10)
20	S (560)							L(26)		G(12)
21	E (570)							D(27)		L(9)
22	S (260)							V(12)		V(5)
23	S (130)							Y(5)		L(7)
24	F (280)									D(11)
25	S (-43)									V(5)
26	Q (210)									Y(6)
27	C (190)									T(8)
28	S (40)									Y(1)
29	K (140)									A(2)
30	D (80)									N(3)
31	S (57)									G(2)
32	G (72)									F (3)
33	Y (84)									S (4)
34	S (25)									S (2)
35	M(43)									–
36	L (35)									C(1)
37	T (11)									A(1)
38	A (21)									
39	T (11)									
40	A (18)									
41	L (20)									
42	P (11)									
43	T (14)									
44	N (14)									
45	A (8)									
46	Q (9)									
47	Y (5)									
48	K (4)									
49	L (5)									
50	M(4)									
51	C (8)									
52	A (8)									

N-term, sequencing of the N-terminal end of the *S*-carboxymethylated cinnamomin. Values in parentheses indicate the differential amount (pmol) of phenylthiohydantoin amino acids determined at each step vs the former. Amino acids are indicated using the single-letter code. (□) Indicates that the sequencing process has been manually stopped, (*) that the corresponding residue has been deamidated. A, B and N-term were separated on a C8 Aquapore RP 300 Brownlee™ column, while tryptic peptides were chromatographed on a C8 Zorbax™ column

3.4.21.4) treated with L-1-(tosylamido)-2-phenylethylchloromethylketone (enzyme to substrate weight ratio, 1 : 100) for 3 h at 37°C. The digestion was stopped by bringing the pH up to 2-3 with 6 N HCl. All digested materials were lyophilized prior to further separation.

Peptide fractionations by RPLC were conducted with an SP 8700 Spectra Physics equipment and monitored with a Jasco UV diode array detector. Tryptic peptides were separated at 40°C on a C8 Zorbax™ 80 × 6.2 mm column at a flow rate of 1 ml · min^{-1}. A gradient

from 0 to 100% of solvent B was applied in 60 min; solvent A was Millipore™-treated water with 0.1% CF_3COOH and solvent B 40% Millipore™-treated water, 60% CH_3CN (FISONS, far UV grade) and 0.09% CF_3COOH. Asp-N peptides were obtained at room temperature with a C8 Aquapore RP 300 Brownlee™ 30 × 4.6 mm column at a flow rate of 0.5 ml · min^{-1}. A gradient from 0 to 100% of solvent B was applied in 60 min; in this case solvent B was 40% water, 60% CH_3CN and 0.09% CF_3COOH. CNBr peptides were separated with the same system; peptide B was rechromatographed at room temperature with a C8 Aquapore RP 300 Brownlee™ 30 × 2.1 mm column at a flow rate of 0.2 ml · min^{-1}. The following gradient was used: from 0 to 20% of solvent B in 5 min, then from 20% to 80% of solvent B in 55 min; solvent A was 5% CH_3CN in 25 mM CH_3COONH_4, pH 7.2, and solvent B 50% CH_3CN in 50 mM CH_3COONH_4, pH 7.2.

2.3. *Protein sequencing and flexibility and hydropathy profile determination*

Automated Edman degradation of the whole protein and of peptides was performed following the original method of Hewick et al. [8] using an Applied Biosystems 475A sequencer and its on line phenyl-thiohydantoin-amino acid analyzer model 120A with reagents and methods of the manufacturer. The hydropathy profiles were done with the programme of Kyte and Doolittle [9] and flexibility diagrams obtained with the method of Karplus and Schulz [10].

3. RESULTS

3.1. *Isolation and sequencing of cinnamomin peptide fragments*

Table 1 illustrates the sequencing data of cinnamomin and the derived peptides noted T (numbered according to their RPLC elution position), B and A for tryptic, CNBr and Asp-N cleavage products, respectively. With an initial sequencing yield of 45%, the *S*-carboxymethylated N-terminal end was sequenced up to Ala52, which allowed the alignment of peptides T3, T6, T5 and T4. The sequencing of peptide B was sufficient to align the C-terminal end of the molecule (T4, T7, T1 and T2). Peptide A, obtained by partial cleavage at the amino side of the aspartic acid corresponding to a limited deamidation form of Asn89, confirmed the alignment of T_1 and T_2 and the C-terminus. Except that deamidation, no microheterogeneity was observed in the sequence of cinnamomin.

3.2. *Amino acid sequence of cinnamomin*

The alignment of the sequenced peptides of cinnamomin is shown in fig.1. Like cryptogein and capsicein, cinnamomin lacked some amino acids (Trp, His, Arg). It exhibited the same number of residues (98) and a similar M_r (10 282 instead of 10 323 for cryptogein and 10 155 for capsicein). Its calculated isoelectric point was 7.85, close to that of cryptogein because of the same number of lysyl residues (the calculated theoretical isoelectric points are 8.49 for cryptogein and 4.54 for capsicein).

3.3. *Comparison of the structures of cinnamomin, cryptogein and capsicein*

The 3 elicitins were homologous to each other and no deletion was necessary for aligning their sequences (fig.2). The percent match between cinnamomin and cryptogein was 87.8% and only 82.7% between cinnamomin and capsicein. The better homology with cryptogein was clearly observed in the N-terminal end

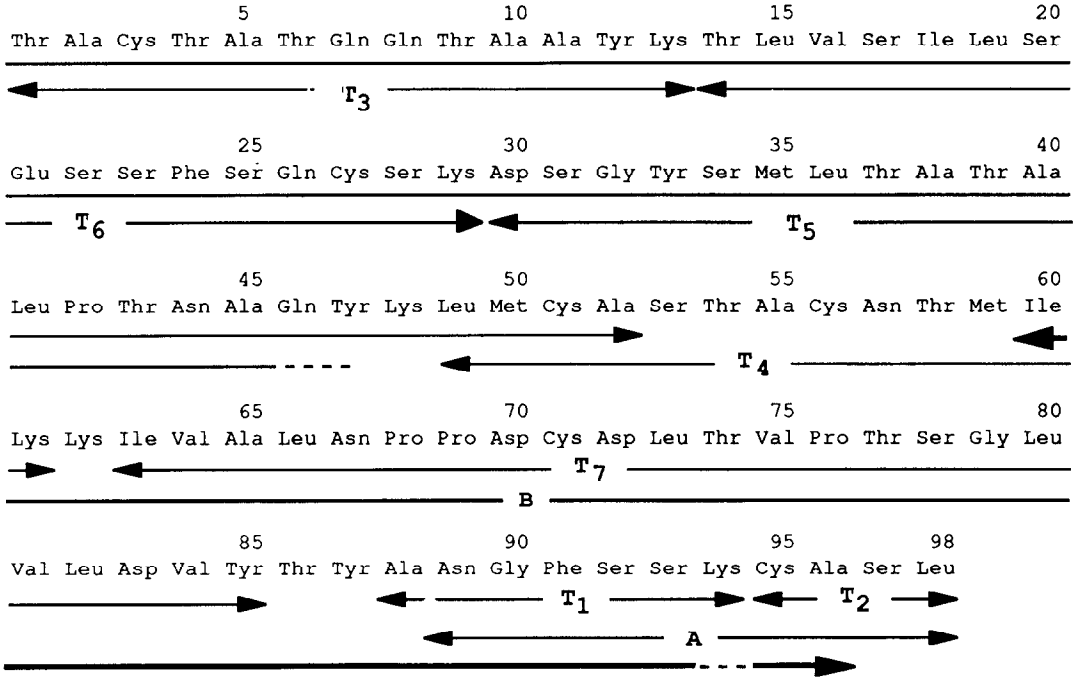

Fig.1. Complete amino acid sequence of cinnamomin secreted by *Phytophthora cinnamomi*. (→) N-terminus of the *S*-carboxymethylated protein; (←→) cleaved peptides (T, tryptic peptides; B, CNBr-cleaved peptides; A, endoprotease Asp-N-cleaved peptides). Dashed lines indicate undetermined residues.

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                    5                  10                  15                  20
Cry ──► Thr Ala Cys Thr Ala Thr Gln Gln Thr Ala Ala Tyr Lys Thr Leu Val Ser Ile Leu Ser
Cin ──► Thr Ala Cys Thr Ala Thr Gln Gln Thr Ala Ala Tyr Lys Thr Leu Val Ser Ile Leu Ser
Cap ──► [Ala Thr] Cys Thr [Thr] Thr Gln Gln Thr Ala Ala Tyr [Val Ala] Leu Val Ser Ile Leu Ser

                    25                  30                  35                  40
Cry ──► Asp Ala Ser Phe Asn Gln Cys Ser Thr Asp Ser Gly Tyr Ser Met Leu Thr Ala Lys Ala
Cin ──► [Glu] [Ser] Ser Phe [Ser] Gln Cys Ser [Lys] Asp Ser Gly Tyr Ser Met Leu Thr Ala [Thr] Ala
Cap ──► Asp [Ser] Ser Phe Asn Gln Cys [Ala] Thr Asp Ser Gly Tyr Ser Met Leu Thr Ala [Thr] Ala

                    45                  50                  55                  60
Cry ──► Leu Pro Thr Thr Ala Gln Tyr Lys Leu Met Cys Ala Ser Thr Ala Cys Asn Thr Met Ile
Cin ──► Leu Pro Thr [Asn] Ala Gln Tyr Lys Leu Met Cys Ala Ser Thr Ala Cys Asn Thr Met Ile
Cap ──► Leu Pro Thr Thr Ala Gln Tyr Lys Leu Met Cys Ala Ser Thr Ala Cys Asn Thr Met Ile

                    65                  70                  75                  80
Cry ──► Lys Lys Ile Val Thr Leu Asn Pro Pro Asn Cys Asp Leu Thr Val Pro Thr Ser Gly Leu
Cin ──► Lys Lys Ile Val [Ala] Leu Asn Pro Pro [Asp] Cys Asp Leu Thr Val Pro Thr Ser Gly Leu
Cap ──► [Thr] Lys Ile Val [Ser] Leu Asn Pro Pro [Asp] Cys [Glu] Leu Thr Val Pro Thr Ser Gly Leu

                    85                  90                  95          98
Cry ──► Val Leu Asn Val Tyr Ser Tyr Ala Asn Gly Phe Ser Asn Lys Cys Ser Ser Leu
Cin ──► Val Leu [Asp] Val Tyr [Thr] Tyr Ala Asn Gly Phe Ser [Ser] Lys Cys [Ala] Ser Leu
Cap ──► Val Leu Asn Val Tyr Ser Tyr Ala Asn Gly Phe Ser [Ala] [Thr] Cys [Ala] Ser Leu
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Fig.2. Comparison of the amino acid sequences of cinnamomin, cryptogein and capsicein. Cry, cryptogein; Cin, cinnamomin; Cap, capsicein. Boxes emphasize differences.

up to residue 21. Most of the replacements were conservative [11]. With respect to cryptogein, 4 mutations were identical to those found in capsicein, for instance at positions 22 (Ala replaced by Ser), 39 (Lys replaced by Thr), 70 (Asn replaced by Asp) and 96 (Ser replaced by Ala), while the 8 others are peculiar to cimmanomin (at positions 21, 25, 29, 44, 65, 83, 86 and 92). Like in cryptogein and capsicein sequences [5], internal se-

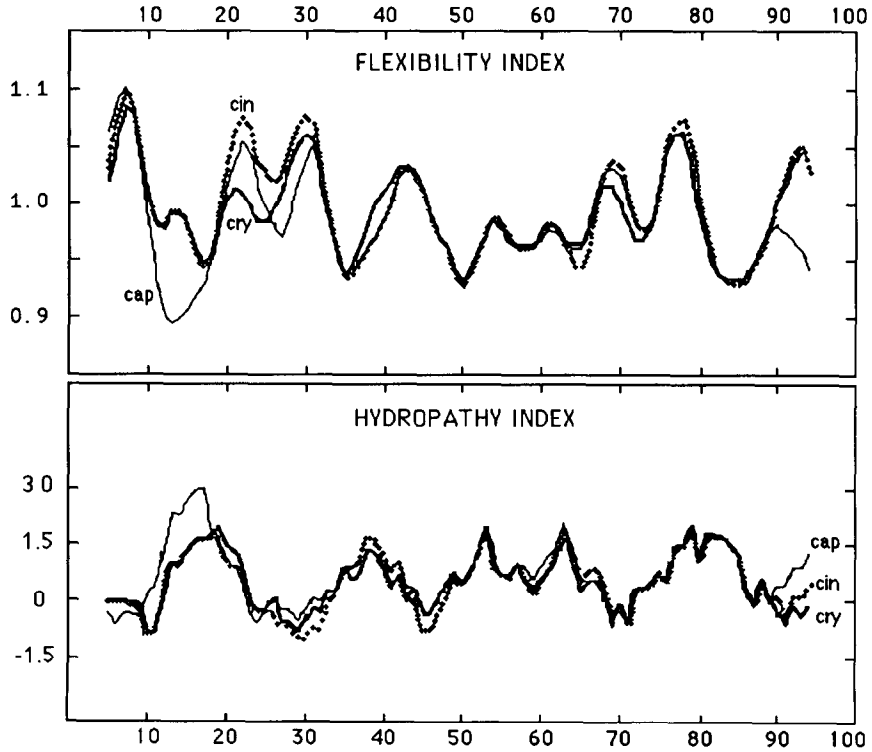

Fig.3. Buried and exposed areas of cinnamomin, cryptogein and capsicein. Profiles were obtained with a window width of 7. Flexibility diagrams were obtained with the method of Karplus and Schulz [10] and hydropathy profiles computed with the programme of Kyte and Doolittle [9]. Bold lines correspond to cryptogein (cry), dashed lines to cinnamomin (cin), the others to capsicein (cap). All computations were done using the similarity matrix of Dayhoff et al. [11].

quence homologies consisting of the irregular repetition of 2 common consensus sequences of 6 to 7 residues occurred in cinnamomin.

The 3 flexibility and hydropathy profiles (fig.3) were very similar to each other, except at the C-terminal end and around position 15 where differences were prominent, while the 20–30 region was variable. In the 2 terminal regions, only capsicein showed differences, while cinnamomin and cryptogein profiles were very close together. Cinnamomin, with an average hydropathy index of 0.18 was comparable to cryptogein (0.13) but more hydrophilic than capsicein (0.39). The flexibility profiles reveal 6–7 putative antigenic determinants; only the C-terminal one was not predicted in capsicein and could be expected to differentiate it from the 2 other elicitins. The comparison of these diagrams with the primary structure showed that only 2 regions were to be involved in the difference of biological activity found between these molecules: an N-terminal region around position 13 where Lys in cinnamomin and cryptogein was replaced by Val in capsicein and a second one at the C-terminal end around position 94 involving the change of Lys in cinnamomin and cryptogein by Thr in capsicein.

4. DISCUSSION

With the exception of Asn89 which was partly deamidated, likely in the course of sample preparation, the absence of microheterogeneity all along the cinnamomin sequence establishes that cinnemomin, like other elicitins [5], arises from a single gene. Not only the amino acid sequences of cinnamomin and cryptogein were more homologous to each other than to capsicein, but they exhibit the same levels of biological activities [1,3,4]. Since the central core of the 3 molecules was highly conserved, it was likely essential for both biological activities. The terminal ends which vary from one elicitin to another would modulate differently these activities. Among the 15 replacements observed between cryptogein and capsicein, only those occurring at positions 13, 39, 61, 93 and 94 were not conservative. If one were to correlate the amino acid replacements with the biological activities, it is likely that neither the 5 replacements common to cinnamomin and capsicein nor those which were peculiar to cinnamomin were essential to explain the differences in biological activities, namely a reduction in remote leaf necrosis and an enhancement in the induction of the protection against *Phytophthora nicotianae* [5]. The only potential candidates were found at 9 positions: 1 (Thr replaced by Ala), 2 and 5 (Ala by Thr), 13 (Lys by Val), 14 (Thr by Ala), 28 (Ser by Ala), 61 (Lys by Thr), 72 (Asp by Glu) and 94 (Lys by Thr). The inversion between positions 1 and 2 and the mutations at positions 5 and 14, as well as those occurring at positions 28 and 72, were conservative [11]. Consequently, the replacements which are likely involved in the control of the level of necrotic activity with respect to the protective role of elicitins were restricted to only 3 positions (13, 61 and 94) instead of 5, as previously published [5]. In every case, a lysyl residue was involved.

When adding the comparison of the hydropathy plots, the role of Lys61 (which is next to another lysyl residue) appeared to be of lesser importance than the 2 strategic residues 13 and 94. As leaf symptoms occur at a distance from the application court and involve a systemic elicitin migration, differences in necrotic activity might also relate to features affecting the migration properties: not only are cinnamomin and cryptogein more hydrophilic than capsicein, but also their positive net charge would favour the negatively charged cell-wall crossing.

These results confirm more precisely those already inferred from the comparison of the sequences and deduced features of cryptogein and capsicein [5]. This information will be essential to engineer cryptogein-like molecules devoid of their necrotic properties to synthesize protective biochemicals against *Phytophthora*.

Acknowledgements: This study was supported by an INRA Action Incitative Programmée no. 89/4697. The authors wish to thank Dr Maud Bruneteau (Laboratoire de Biochimie microbienne, Université Claude Bernard-Lyon I) for providing cinnamomin and Monique Mansion for her skilful technical assistance.

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