Fatty acids bind to the fungal elicitor cryptogein and compete with sterols

H. Osman[a], V. Mikes[b], M.-L. Milat[a], M. Ponchet[c], D. Marion[d], T. Prangé[e], B.F. Maume[a], S. Vauthrin[a], J.-P. Blein[a,*]

[a] *UMR 692 INRA, Université de Bourgogne, Laboratoire de Phytopharmacie et de Biochimie des Interactions Cellulaires, INRA, BP 86510, 21065 Dijon Cedex, France*
[b] *Department of Biochemistry, Faculty of Science, Masaryk University, Kotlarska 2, 61137 Brno, Czech Republic*
[c] *IPMSV, INRA, BP 2078, 06606 Antibes Cedex, France*
[d] *Laboratoire de Biochimie et Technologie des Protéines, INRA, BP 71627, 44316 Nantes Cedex 03, France*
[e] *LCRB, Faculté de Pharmacie, 4 av. de l'Observatoire, 75270 Paris Cedex 06, France*

Received 27 October 2000; revised 27 December 2000; accepted 27 December 2000

First published online 10 January 2001

Edited by Shozo Yamamoto

Abstract Cryptogein is a proteinaceous elicitor of plant defense reactions which also exhibits sterol carrier properties. In this study, we report that this protein binds fatty acids. The stoichiometry of the fatty acid–cryptogein complex is 1:1. Linoleic acid and dehydroergosterol compete for the same site, but elicitin affinity is 27 times lower for fatty acid than for sterol. We show that C7 to C12 saturated and C16 to C22 unsaturated fatty acids are the best ligands. The presence of double bonds markedly increases the affinity of cryptogein for fatty acids. A comparison between elicitins and known lipid transfer proteins is discussed.

Key words: Elicitin; Lipid–protein interaction; Fatty acid; Sterol; *Phytophthora*

1. Introduction

The transfer of lipidic molecules (fatty acids (FAs), phospholipids, sterols) is an important process occurring in all living organisms (for review see [1,2]), and eukaryotic cells contain several lipid binding and -carrier proteins.

To date, there is no evidence that phospholipid or phytosterol transport in plant cells requires carrier proteins [3]. However, plants contain proteins which are able to facilitate the transfer of lipids (for review see [4]). They are small, mainly extracellular proteins able to bind non-specifically FAs, acyl-CoA esters, phospholipids and even prostaglandins [5]. They could play a crucial role in plant defense mechanisms [6] since some antifungal or antimicrobial proteins show homology with plant non-specific lipid transfer proteins [7–9], and transgenic overexpression of the barley LTP2 protein, in tobacco and *Arabidopsis*, enhances tolerance to pathogens [10]. Moreover, plants are able to react to spores of fungal pathogens, with responses similar to those elicited by chitin fragments, via a detection of fungal sterols [11]. In addition, unsaturated FAs, for example linoleic acid, induce systemic resistance of potato against *Phytophthora infestans* [12]. However, the mechanisms involved in the induction of systemic resistance in plants by sterols or FAs are not known.

We previously reported that elicitins, proteins secreted by the phytopathogenic *Phytophthora* sp., are elicitors of plant defense reactions [13,14]. They bind putative receptors, located on the plasma membrane, and trigger the now well documented signal transduction pathway involved in plant cell–elicitor interactions (for recent review see [15]). Elicitins have also been characterized as extracellular sterol binding proteins [16,17]. They are able to bind sterols and to catalyze their transfer between micelles, artificial membranes, and between micelles and natural membranes [18]. Using dehydroergosterol (DHE), a fluorescent sterol, we demonstrated that all the elicitins interact with sterols in the same way but with some time-dependent differences. They have the same binding stoichiometry, i.e. one sterol molecule bound per elicitin molecule and their dissociation constants are in the same range (0.11–0.58 μM), although these proteins exhibit different rates of sterol exchange [17]. Moreover, the three-dimensional structure of an ergosterol–K13H–cryptogein complex has been reported [19]. It shows that sterol is enclosed in a hydrophobic cavity of this small (10 kDa) basic protein. Thus, elicitins can be considered as a new class of sterol carrier proteins (SCPs).

In this paper, the interaction between cryptogein, an elicitin secreted by *P. cryptogea*, and FAs is presented.

2. Materials and methods

All experiments were performed in triplicate and results are expressed with standard error bars.

2.1. Chemicals, elicitors and plant materials

DHE and FAs (Sigma-Aldrich Co.) were dissolved in ethanol. Before each titration experiment, the concentration of DHE was spectrophotometrically determined. Cryptogein was obtained as previously described [13], dissolved in water or in an appropriate buffer, and stored at −20°C.

2.2. Isolation of the cryptogein–FA complexes

A cryptogein–palmitic acid complex was obtained by a previously reported chromatographic method [17], after minor modifications.

*Corresponding author. Fax: (33)-03-80 69 32 65.
E-mail: blein@chassagne.epoisses.inra.fr

Abbreviations: DHE, $\Delta^{5,7,9(11)22}$-ergostatetraen-3β-ol (dehydroergosterol); FA, fatty acid; FABP, fatty acid binding protein; SCP, sterol carrier protein

PII: S0014-5793(01)02078-6

Briefly, to cryptogein (1 mg) in 1 ml buffer I, containing 175 mM mannitol, 0.5 mM $CaCl_2$, 0.5 mM K_2SO_4, and 5 mM MES, pH 5.75, the ethanolic solution of palmitic acid was added dropwise (2 mol lipid/mol cryptogein). After incubation (10 min), the complex was isolated from the excess of lipid by chromatography on a Sephadex G-25 (Pharmacia) column (125 mg equilibrated with buffer I). From the eluted fractions, the amounts of cryptogein were assessed using Lowry's method [20], and palmitic acid was extracted three times with dichloromethane and esterified. The corresponding methyl ester was analyzed by gas chromatography using a Perkin Elmer Autosystem equipped with a Supelco Sac-5 column (30 m × 0.25 mm × 0.25 μm), a FID detector and a PE Nelson model 1020 computer. Gas flows were set at 1, 45 and 450 ml/min for nitrogen, hydrogen and air, respectively. The temperature program was as follows: 50 to 285°C, 45°C/min, 285°C for 30 min. Quantitation was performed using a calibration curve obtained with palmitic methyl ester and 5α-cholestane as internal standard.

2.3. Interaction between cryptogein, DHE and FAs

Fluorescence measurements were performed at 25°C with a Shimadzu RF 5301 PC spectrofluorimeter. The excitation and emission wavelengths were set at 325 and 370 nm, respectively. DHE and FAs were incubated, in a stirred cuvette containing 2 ml of buffer I at pH 7.0, during 1 min and the fluorescence was recorded (F_0). Cryptogein was then added and, after 1 min, the fluorescence was recorded (F). Results are expressed either as percentage of the fluorescence of the cryptogein–DHE complex according to $((F-F_0)/F_c)100$, where F_c is the fluorescence of the DHE–cryptogein complex in absence of FAs, or as fluorescence ($F-F_0$) in arbitrary units. The fluorescence of cryptogein was negligible [18].

3. Results

To investigate the affinity of FAs for cryptogein, the protein was incubated with DHE and various FAs.

3.1. Effect of linoleic acid on the cryptogein–DHE complex stability

When cryptogein was added to a mixture of DHE and linoleic acid, the fluorescence of the cryptogein–DHE complex decreased. This effect was proportional to the linoleic acid concentration in the range 0–18 μM. At the highest concentration of the FA, about 17% of the initial fluorescence remained (Fig. 1). The IC_{50} was 5.4 ± 0.8 μM. These observations could result either from a change in the cryptogein conformation induced by the linoleic acid or from competition between sterol and FA.

We tested the effect of increasing linoleic acid concentrations on the relative fluorescence of the cryptogein–DHE complex, at five different DHE concentrations. The fluorescence quantum yield of free DHE is much lower than that of bound DHE. Moreover, the experiments were performed at low bound DHE concentrations. In these conditions, results could be expressed as Lineweaver–Burk and Dixon plots (Fig. 2A,B, respectively). All straight lines were obtained by linear regression (c.r. = 0.97–0.99). The Lineweaver–Burk plots intercepted the ordinate axis at the same point (0.01378 ± 0.00084, Fig. 2A). The apparent K_d for DHE (0.126 μM) was determined from the curve without FA. These curves are representative of a competitive inhibition phenomenon. In such kinetics, a replot of the slope of each reciprocal plot versus the corresponding inhibitor concentration ([I]) is a straight line and the intercept on the abscissa axis is $-K_i$ (K_i = 3.5 μM, c.r. = 0.99, Fig. 2A, inset). This linear representation indicated a pure competitive inhibition.

The Dixon plot confirmed this type of inhibition and the determination of the K_i value, which was read as the −[I] value corresponding to the intersection of the lines (3.5 μM, Fig. 2B). Moreover, replot of the slopes of the Dixon plot versus 1/DHE should be a straight line through the origin (c.r. = 0.99, Fig. 2B, inset). Thus, both representations clearly state that linoleic acid competed with DHE for the same cryptogein binding site.

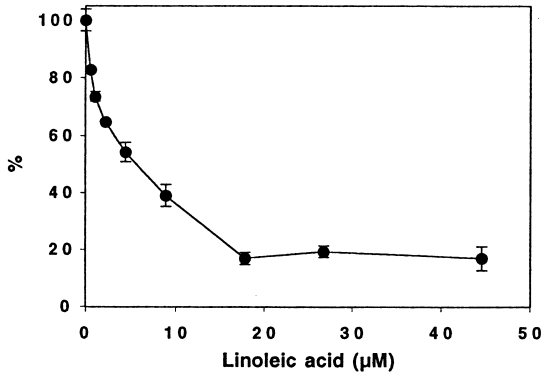

Fig. 1. Effect of linoleic acid on the cryptogein–DHE complex stability. DHE (100 nM) and linoleic acid were incubated together for 1 min, and then cryptogein (250 nM) was added. Results are expressed as a percentage of the fluorescence of the cryptogein–DHE complex. Experiments were performed in triplicate and results are expressed with standard error bars.

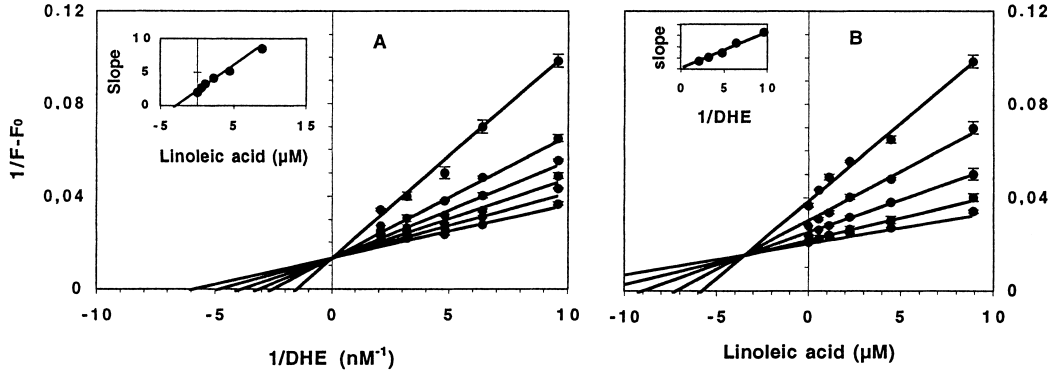

Fig. 2. Competition between DHE and linoleic acid to bind to cryptogein. Cryptogein (250 nM) and DHE were incubated together for 1 min, and then linoleic acid was added. (A) The $1/F-F_0$ versus 1/DHE concentrations in the presence of different fixed concentrations of linoleic acid. Inset: replot of the slopes of the reciprocal plot versus the linoleic acid concentrations. (B) Dixon plot of the precedent data. Inset: replot of the slopes of the Dixon plot. Experiments were performed in triplicate and results are expressed with standard error bars.

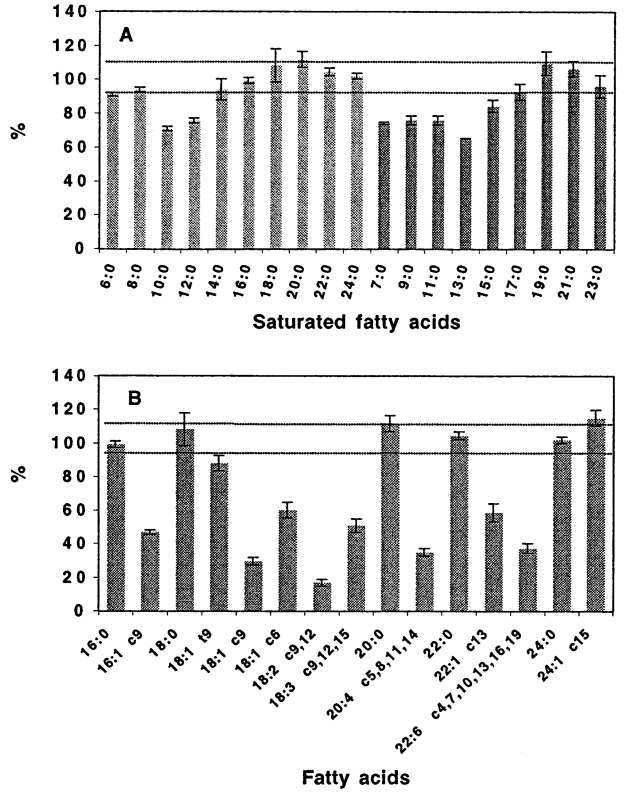

Fig. 3. Effect of FAs on the cryptogein–DHE complex stability. Cryptogein (250 nM) and DHE (100 nM) were incubated together for 1 min, and then FAs (18 μM) were added. Results are expressed as a percentage of the fluorescence of the cryptogein–DHE complex. Experiments were performed in triplicate and results are expressed with standard error bars. Dotted lines represent the standard error of the control.

3.2. *Effect of the FA structures on the cryptogein–DHE complex stability*

To check the affinity of natural FAs for cryptogein, competition between FAs and DHE was studied. Two groups were used: the saturated FAs with 6–24 carbon chain lengths and unsaturated FAs with 16–24 carbons and different numbers and geometry of unsaturation. The FA concentration was 18 μM, which corresponds to the concentration which efficiently displaced DHE in the previous experiment (Fig. 1).

Some saturated FAs weakly competed with DHE (Fig. 3A): even FAs with 10–12 carbons (20–25%), and odd FAs with a 7–15 carbon chain length (17–36%). Surprisingly, most of common saturated FAs were not able to displace the DHE from the elicitin. Nevertheless, a cryptogein–palmitic acid (16:0) complex was obtained using the method reported for the sterol–cryptogein complexes [17]. The total protein recovery after chromatography was 82.7 ± 2.3% and the stoichiometry of the complex was 1.01 ± 0.06, indicating that palmitic acid binds cryptogein despite exhibiting no competition with DHE (Fig. 3A).

The affinities of cryptogein for various FAs with single or more double bonds (*cis* or *trans*) were compared (Fig. 3B). The *cis*-9 position was the most efficient (54 and 70% for $16{:}1\Delta^{9cis}$ and $18{:}1\Delta^{9cis}$, respectively) in contrast to the *trans*-9 or the *cis*-6 positions (12% for $18{:}1\Delta^{9trans}$, 40% for $18{:}1\Delta^{6cis}$). Linoleic acid ($18{:}2\Delta^{9,12cis}$) was the best competitive molecule (83%) whereas linolenic acid ($18{:}3\Delta^{9,12,15cis}$) was markedly less efficient (49%). Except for C24, all the results confirm that the unsaturation reinforced the affinity of these lipids for cryptogein.

4. Discussion

The results presented in this paper show that cryptogein can bind not only sterols but also FAs. However, the affinity for sterols is 27 times higher. The binding and carrier properties of elicitins are similar to those of SCP-2 that binds sterols and FAs with a decreasing order of magnitude [21,22] although their structures and cellular localizations are different. The FA–elicitin interactions are very specific, since the carbon chain length, and the presence and the position of double bonds, strongly affect the FA affinity for cryptogein. These characteristics are in accordance with those reported for brain FA binding protein (FABP) [23]. Nevertheless, SCP-2 can stimulate intermembrane sterol transfer by direct membrane interaction [24] and the sterol–elicitin complex has been isolated [17] and characterized by X-ray diffraction analyses [19]. Finally, while linoleic acid competed with cholesterol for the same binding site, apparently palmitic acid bound to cryptogein when added alone, but it was unable to compete with sterol binding. This could suggest that there are two binding sites for FAs, one of which can also bind sterols. This is precedent for this situation in the mammalian liver FABP (l-FABP) and SCP-2: nuclear magnetic resonance (NMR) data show that l-FABP binds two FAs but only one sterol [25], and in SCP-2, NMR data show the existence of two ligand binding sites, one of which binds both sterol and FA, while the other binds only FA [26]. Further experiments are needed to determine the number of FA binding sites in the case of elicitins.

However, FA interactions with elicitins differ from those observed with maize LTP [27]. nsLTP1 is the best characterized group of plant LTPs with basic p*I*, conserved cysteine pattern and a molecular mass of about 9 kDa. LTPs are able to transport phospholipids and to bind FAs, showing either narrow [23,28] or broad specificity [5,27] towards the nature of the transferred lipids. Moreover, despite a high degree of sequence identity and close global folding [29], LTPs can exhibit structural differences leading to different affinities for lipids [30]. Structures of plant nsLTP complexed with palmitic acid demonstrated a 1:1 molar ratio [31,32] or 2:1 (lipid/protein) [27,30,33]. However, these proteins do not bind sterol [1] and their role is not fully understood. They could be involved in cuticle formation, pathogen defense reactions or responses to environmental changes [4]. Recently, the structure of a wheat nsLTP1 complexed with two molecules of phospholipid, inserted head to tail has been reported [33]. In this structure, the cavity described in the lipid-free protein becomes a tunnel. In contrast, the structures of void cryptogein [34] or of an ergosterol–cryptogein complex [19] indicate that the hydrophobic core remains a cavity, despite conformational changes. These structural differences between nsLTP and elicitins could be at the origin of the lipid specificity observed. Altogether, these data reveal the complexity of the LTP family and the wide role played by these proteins.

Elicitins are extracellular proteins secreted by *Phytophthora* sp. The role of these proteins in *Phytophthora* metabolism is the subject of much speculation. As they can pick up sterols from plant membranes [18], they could be involved in fungal

development. Moreover, lipidic molecules can also play a key-role during the early phases of *Phytophthora* sp. parasitism. In this way, elicitin interactions with structure-containing lipidic material such as cuticles, suberized walls and biological membranes will need a particular attention, to determine whether signal recognition between plant and pathogen could originate from this particular protein–lipid interaction. At the membrane level, lipidic microdomains could be involved, as it was recently reported in pathogen– or toxin–lipid raft interactions [35].

Acknowledgements: This work was supported by INRA, SEITA-ARN (Société d'Exploitation Industrielle des Tabacs et Allumettes-Association de Recherche sur les Nicotianées) and by grants from the Conseil Régional de Bourgogne, from the MENRT (Ministère de l'Education Nationale, de la Recherche et de la Technologie), and from Barrande no. 99002. We thank M.-J. Farmer for revising the English.

References

[1] Wirtz, K.W.A. (1991) Annu. Rev. Biochem. 60, 73–99.
[2] Bourgis, F. and Kader, J.-C. (1997) Physiol. Plant 100, 78–84.
[3] Moreau, P., Bessoule, J.J., Mongrand, S., Testet, E., Vincent, P. and Cassagne, C. (1998) Prog. Lipid Res. 37, 371–391.
[4] Kader, J.-C. (1996) Ann. Rev. Plant Physiol. Plant Mol. Biol. 47, 627–654.
[5] Tassin-Moindrot, S., Caille, A., Douliez, J.P., Marion, D. and Vovelle, F. (2000) Eur. J. Biochem. 267, 1117–1124.
[6] Garcia-Olmedo, F., Molina, A., Segura, A. and Moreno, M. (1995) Trends Microbiol. 3, 72–74.
[7] Terras, F.R.G., Goderis, I.J., Van Leuven, F., Van derleyden, J., Cammue, B.P.A. and Broekaert, W.F. (1992) Plant Physiol. 100, 1055–1058.
[8] Nielsen, K.K., Nielsen, J.E., Madrid, S.M. and Mikkelsen, J.D. (1996) Plant Mol. Biol. 31, 539–552.
[9] Cammue, B.P.A. et al. (1995) Plant Physiol. 109, 445–455.
[10] Molina, A. and Garcia-Olmedo, F. (1997) Plant J. 12, 669–675.
[11] Granado, J., Felix, G. and Boller, T. (1995) Plant Physiol. 107, 485–490.
[12] Cohen, Y., Gisi, U. and Mosinger, E. (1991) Physiol. Mol. Plant Pathol. 38, 255–263.
[13] Bonnet, P., Bourdon, E., Ponchet, M., Blein, J.-P. and Ricci, P. (1996) Eur. J. Plant Pathol. 102, 181–192.
[14] Keller, H., Blein, J.P., Bonnet, P. and Ricci, P. (1996) Plant Physiol. 110, 365–376.
[15] Ponchet, M. et al. (1999) Cell. Mol. Life Sci. 56, 1020–1047.
[16] Mikes, V., Milat, M.-L., Ponchet, M., Ricci, P. and Blein, J.-P. (1997) FEBS Lett. 416, 190–192.
[17] Mikes, V., Milat, M.-L., Ponchet, M., Panabières, F., Ricci, P. and Blein, J.-P. (1998) Biochem. Biophys. Res. Commun. 245, 133–139.
[18] Vauthrin, S., Mikes, V., Milat, M.-L., Ponchet, M., Maume, B., Osman, H. and Blein, J.-P. (1999) Biochim. Biophys. Acta 1419, 335–342.
[19] Boissy, G., O'Donohue, M., Gaudemer, O., Perez, V., Pernollet, J.C. and Brunie, S. (1999) Protein Sci. 8, 1191–1199.
[20] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[21] Crain, R.C. and Zilversmit, D.B. (1980) Biochemistry 19, 1440–1447.
[22] Avdulov, N.A., Chochina, S.V., Igbavboa, U., Warden, C.S., Schroeder, F. and Wood, W.G. (1999) Biochim. Biophys. Acta 1437, 37–45.
[23] Balendiran, G.K. et al. (2000) J. Biol. Chem. 275, 27045–27054.
[24] Woodford, J.K., Colles, S.M., Myers-Payne, S., Billheimer, J.T. and Schroeder, F. (1995) Chem. Phys. Lipids 76, 73–84.
[25] Cistola, D.P., Sacchettini, J.C., Banaszak, L.J., Walsh, M.T. and Gordon, J.I. (1989) J. Biol. Chem. 264, 2700–2710.
[26] Stolowich, N., Frolov, A., Petrescu, A.D., Scott, A.I., Billheimer, J.T. and Schroeder, F. (1999) J. Biol. Chem. 274, 35425–35433.
[27] Zachowski, A., Guerbette, F., Grosbois, M., Jolliot-Croquin, A. and Kader, J.C. (1998) Eur. J. Biochem. 257, 443–448.
[28] Dansen, T.B., Westerman, J., Wouters, F., Wanders, R.J.A., Van Hoek, A., Gadella, T.W.J. and Wirtz, K.W.A. (1999) Biochem. J. 339, 193–199.
[29] Poznanski, J., Sodano, P., Suh, S.W., Lee, J.Y., Ptak, M. and Vovelle, F. (1999) Eur. J. Biochem. 259, 692–708.
[30] Guerbette, F., Grosbois, M., Jolliot-Croquin, A., Kader, J.C. and Zachowski, A. (1999) Biochemistry 38, 14131–14137.
[31] Shin, D.H., Lee, J.Y., Hwang, K.Y., Kim, K.K. and Suh, S.W. (1995) Structure 3, 189–199.
[32] Lerche, M.H. and Poulse, F.M. (1998) Protein Sci. 7, 2490–2498.
[33] Charvolin, D., Douliez, J.P., Marion, D., Cohen-Addad, C. and Pebay-Peyroula, E. (1999) Eur. J. Biochem. 264, 562–568.
[34] Boissy, G., de La Fortelle, E., Kahn, R., Huet, J.C., Bricogne, G., Pernollet, J.C. and Brunie, S. (1996) Structure 4, 1429–1439.
[35] Fivaz, M., Abrami, L. and van der Goot, F.G. (2000) Protoplasma 212, 8–14.