Physiol. **20**, 195 (1974).
9. J. M. Giebultowicz, R. A. Bell, R. B. Imberski, *ibid.* **34**, 527 (1988).
10. B. J. Thorson and J. G. Riemann, *ibid.* **23**, 1189 (1977).
11. J. G. Riemann and B. J. Thorson, *Ann. Entomol. Soc. Am.* **71**, 575 (1978); J. M. Giebultowicz, unpublished observations.
12. Testis-UVD-SV complexes were dissected with aseptic technique and placed individually in 0.4 ml of Graces insect medium (Gibco) containing 10% fetal bovine serum (Gibco) and 1% antibiotic-antimycotic solution (Sigma) in four-well Nuclon Multidishes. Cultures were kept at 25°C in airtight incubator chambers (Billups-Rothenberg) filled with oxygen and humidified. At designated times 8 to 12 preparations were dissected to determine the presence of sperm bundles in the UVD and SV.
13. Mean number of sperm bundles released during one cycle in intact pharate adult males was 55.2 (SE = 21, n = 8). In cultured testis-UVD-SV complexes the mean number of sperm bundles released was 39.1 (SE = 9, n = 8) during the first cycle in vitro, 16.6 (SE = 10, n = 10) during the second cycle, and 4.3 (SE = 3, n = 8) during the third cycle.
14. Photoperiod rather than temperature is the main entraining factor for the rhythm of sperm release. When pupae were exposed to a 16L:8D cycle and to a cycle of 16 hours at 28°C and 8 hours at 23°C so that the light and temperature cycles were 6 hours out of phase with each other, the timing of sperm release was in phase with the photoperiod, not the temperature cycle.
15. A. Eskin, *Z. Vgl. Physiol.* **74**, 353 (1971).
16. J. G. Riemann and B. J. Thorson, *J. Morphol.* **149**, 483 (1976).
17. S. Omura, *J. Fac. Agric. Hokkaido Imp. Univ.* **38**, 151 (1936); L. Danilova and V. Verejskaya, *Tsitologiya* **13**, 15 (1971).
18. D. M. Phillips, *J. Cell Biol.* **44**, 243 (1970); J. G. Riemann and B. J. Thorson, *Int. J. Insect Morphol. Embryol.* **1**, 11 (1971).
19. J. M. Giebultowicz, R. L. Ridgway, R. B. Imberski, *Physiol. Entomol.*, in press.
20. We thank J. Choi for technical assistance, R. A. Bell for providing insects, and D. Hayes, J. Klun, M. Menaker, T. Page, and M. Young for reading the manuscript. Supported by USDA competitive grant no. 88-37251-3970 to J.M.G.

9 February 1989; accepted 19 July 1989

A Chitin-Binding Lectin from Stinging Nettle Rhizomes with Antifungal Properties

WILLEM F. BROEKAERT,*† JAN VAN PARIJS, FREDERIK LEYNS, HENK JOOS, WILLY J. PEUMANS†

Rhizomes of stinging nettle contain a small-sized lectin that exhibits binding specificity toward chitin. This lectin inhibits growth of several phytopathogenic and saprophytic chitin-containing fungi in vitro. The antifungal action of the nettle lectin differs from the action of chitinases, which are a ubiquitous class of antifungal plant proteins. Moreover, the nettle lectin acts synergistically with chitinase in inhibiting fungal growth. The nettle lectin may be a promising candidate for possible applications in the genetic engineering of disease-resistant crops.

IN SPITE OF THE VAST RESEARCH ON plant lectins, their physiological role remains a matter of controversy (*1, 2*). As early as 1975, an attractive proposal was forwarded suggesting that wheat germ agglutinin (WGA), a chitin-binding lectin from wheat embryos, plays a role in the defense of seedlings against fungal attack (*3*). This notion was based on the observation that WGA inhibits spore germination and hyphal growth of *Trichoderma viride*. Several investigators have proposed arguments supporting such a protective role for WGA (*4*) and other chitin-binding plant lectins (*5, 6*). More recently, however, it was shown that contaminating chitinases, not the lectins themselves, are responsible for the observed in vitro inhibition of fungal growth (*7*). To reopen the debate, we report that a chitinase-free chitin-binding lectin from stinging nettle rhizomes is a potent inhibitor of fungal growth.

Some years ago a monomeric lectin was isolated (by affinity chromatography on chitin) from rhizomes of stinging nettle (*Urtica dioica L.*) (*8*). With a molecular size of 8.5 kD, *Urtica dioica* agglutinin (UDA) is the smallest plant lectin known. UDA is unusually heat and acid resistant, has high contents of cysteine and tryptophane (*8*), exhibits specificity toward *N*-acetyl-D-glucosamine oligomers (*9*), and shows a striking amino acid sequence homology with WGA (*10*).

Experiments indicated that UDA preparations strongly inhibited fungal growth in vitro. To make sure that our UDA preparation was free of contaminating chitinase, the lectin fraction obtained by affinity chromatography on chitin was further purified by ion-exchange chromatography, gel filtration, and affinity chromatography on ovomucoid Sepharose (*11*). Throughout all these purification steps antifungal activity eluted with hemagglutination activity. The final UDA preparation exhibited no detectable chitinase activity when up to 100-μg amounts were tested by the ^{3}H-labeled chitin degradation assay (*12*), which has a detection limit of 20 ng purified chitinase (*13*). Moreover, chitosanase, β-*N*-acetylglucosaminidase, and β-1,3 glucanase activities were negative when 100-μg amounts were employed in the appropriate assays (*14–16*).

To quantify the fungistatic effect of UDA, an assay was used based on the measurement of hyphal growth of germinated spores. Dose-response curves were determined for seven different fungi that contain chitin in their cell walls (*Botrytis cinerea, Collectotrichum lindemuthianum, Phoma betae, Phycomyces blakesleeanus, Septoria nodorum, Trichoderma hamatum*, and *Trichoderma viride*) and one chitin-negative fungus (*Phytophthora erythroseptica*) (*17*) (Fig. 1). Concentrations required for 50% growth inhibition (IC_{50}) of the chitin-containing fungi varied from 20 to 125 μg/ml, whereas the chitin-negative *Phytophthora erythroseptica* was insensitive to

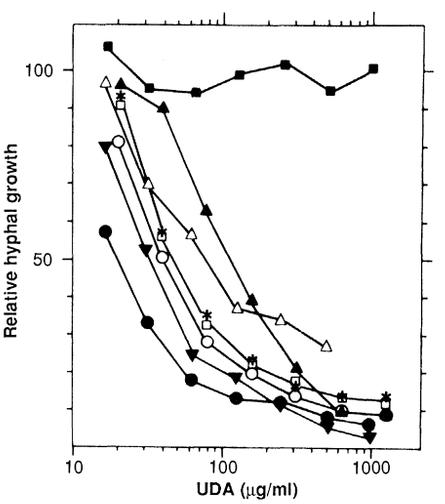

Fig. 1. Antifungal activity of UDA. Hyphal growth of germlings was measured at varying concentrations of UDA (*11*) with the following test organisms (*23*): *B. cinerea* (○), *C. lindemuthianum* (●), *Phoma betae* (△), *Phycomyces blakesleeanus* (▲), *Phytophthora erythroseptica* (■), *S. nodorum* (▼), *T. hamatum* (□), *T. viride* (*). Relative hyphal growth is expressed as percentage of the hyphal growth of control cultures; data are means of three independent experiments. Ratios of standard error to mean were 17% or less. Hyphal growth inhibition assay was performed as follows: 100-μl amounts of spore suspensions (2×10^4 spores/ml) in potato dextrose broth (Difco) were incubated in flat bottom multiwell plates (Nunc) at 22°C until the hyphae of the germlings had an average length of 40 μm. Then, test solutions (50 μl) were added to the germlings, and the plates reincubated at 22°C until the control germlings (50 μl of water added) attained an average length of 500 μm. Average length of 50 individual hyphae was determined from photomicrographs made with an inverted microscope.

W. F. Broekaert, J. Van Parijs, W. J. Peumans, Laboratorium voor Plantenbiochemie, Katholieke Universiteit Leuven, Willem de Croylaan 42, B-3030 Leuven, Belgium.
F. Leyns and H. Joos, Plant Genetic Systems, Jozef Plateaustraat 22, B-9000 Gent, Belgium.

*Present address: F. A. Janssenslaboratorium voor Genetica, Katholieke Universiteit Leuven, Willem de Croylaan 42, B-3030 Leuven, Belgium.
†To whom correspondence should be addressed.

UDA, even at a concentration of 1 mg/ml. None of the eight test fungi, however, was inhibited by any of two other chitin-binding lectins, namely WGA and potato lectin (*18*), when applied at up to 2 mg/ml.

Previously we found that chitinases from various plant sources inhibit growth of *T. hamatum* with an IC_{50} value of approximately 2 μg/ml (*13*), which is about 25 times as low as the IC_{50} of UDA on the same test organism (50 μg/ml). On the other hand, UDA clearly exceeds chitinase in antifungal potency on the phytopathogenic fungus *B. cinerea:* the former has an IC_{50} value of 40 μg/ml, whereas the latter does not affect growth of *B. cinerea* at concentrations lower than 320 μg/ml (*13*). Evidently, UDA and chitinase behave differently with respect to their inhibitory effect on different fungi. Moreover, as can be seen in Fig. 2, the morphological effect of both inhibitory proteins on the fungi is different. For instance, the chitinase from tobacco leaves caused lysis at the hyphal tips of *Phycomyces blakesleeanus*, whereas UDA did not. Furthermore, it was observed that chitinase significantly inhibited the spore germination rate of *T. hamatum* (at concentrations of 8 μg/ml or higher) and *Phycomyces blakesleeanus* (at concentrations of 32 μg/ml or higher) (*13*), whereas UDA did not affect spore germination, even at a concentration of 2 mg/ml (*19*).

To show that the effect of UDA is really due to the lectin, and not to any other kind of contaminating protein, the lectin was subjected to different kinds of inhibitory treatments (Table 1). The antifungal activity of UDA (but also that of tobacco chitinase) could be reversed by addition of the specific inhibitor chitotriose (10 m*M*). UDA and *p*-azophenyl-β-chitobioside–bovine serum albumin (APCB-BSA) formed a specific lectin-glycoconjugate type precipitate (*9*), from which antifungal activity could be recovered. The APCB-BSA reagent, however, did not precipitate tobacco chitinase. Immunoprecipitation of UDA with antiserum to the lectin resulted in almost complete loss of its inhibitory potency, whereas the UDA preparation was not affected by antiserum to tobacco chitinase. In addition, the fungistatic properties of UDA were, like its hemagglutination activity, found to be remarkably heat stable, as they resisted heating for 10 min at up to 75°C. The latter treatment, however, completely destroyed chitinase activity.

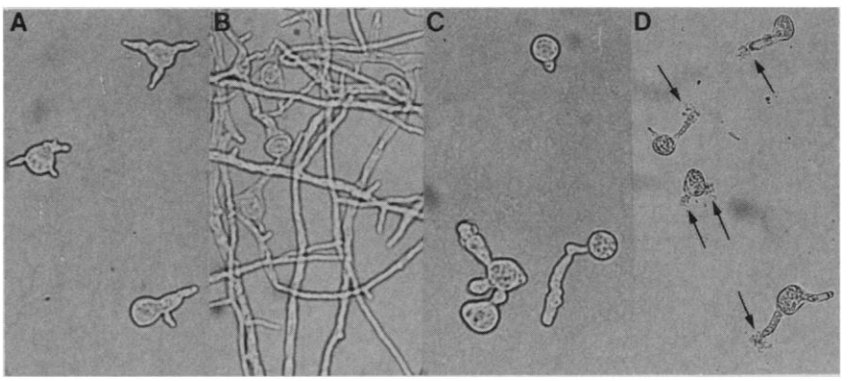

Fig. 2. Comparison of the inhibitory effect of UDA and tobacco chitinase. (**A**) Germlings of *Phycomyces blakesleeanus* at 1 hour after germination (grown as described in the legend to Fig. 1); (**B**) germlings of *P. blakesleeanus* at 8 hours after germination; (**C**) germlings of *P. blakesleeanus* at 8 hours after germination, grown in presence of UDA (600 μg/ml) from 1 to 8 hours after germination; (**D**), germlings of *P. blakesleeanus* at 8 hours after germination, grown in presence of tobacco chitinase (60 μg/ml) (*13*) from 1 to 8 hours after germination. Arrows indicate sites of hyphal lysis.

Because UDA and chitinase affect hyphal growth in a different way, we investigated a possible synergism between both types of fungistatic proteins. As shown in Fig. 3, addition of low concentrations of UDA (which themselves had no effect on hyphal growth) in a 2 to 1 mass ratio to tobacco chitinase, increased the inhibitory potency of the chitinase by about four fold, indicating that lectin and chitinase can act synergistically.

Having established that purified UDA inhibits fungal growth in vitro, we evaluated the antifungal potential of UDA in vivo. An assessment of lectin concentrations in rhizomes and roots of more than 100 individual *Urtica dioica* plants indicated that the lectin concentration in these tissues varies between 0.5 and 3 mg per gram of tissue (fresh weight), which is about one order of magnitude higher than the concentration required for in vitro inhibition of fungal growth. A crude protein extract (UDA, 0.9 mg/ml; total protein, 8 mg/ml) prepared from stinging nettle rhizomes by a previously described extraction procedure (*7*), inhibited growth of the phytopathogenic fungus *B. cinerea* by 85% in the hyphal growth inhibition assay (Fig. 1). However, the same extract from which UDA had been selectively removed by immunoprecipitation with antiserum to UDA or by passage over an

Table 1. Reversibility of antifungal activity of UDA and tobacco chitinase by different treatments. Hyphal growth inhibition assay was performed with *T. hamatum* as described in the legend to Fig. 1. Hemagglutination assays were done with trypsin-treated rabbit erythrocytes (*8*) and chitinase activity was measured radiochemically with ^{3}H-labeled chitin (*12*). Activities are expressed as percentages of activities of untreated samples and are means of three independent experiments. Ratios of standard errors to means were 15% or less. Untreated samples contained 25 μg UDA or 1.25 μg tobacco chitinase in 50 μl 30 m*M* sodium phosphate buffer, *p*H 7.5. Samples treated by immunoprecipitation consisted of 30 μg UDA (or 1.5 μg tobacco chitinase) in 54 μl sodium phosphate buffer and 6 μl antiserum to UDA (or antiserum to tobacco chitinase). The immunoprecipitates formed after standing at 37°C for 1 hour and subsequently at 4°C for 24 hours were removed by centrifugation (10,000*g*, 10 min), and 50 μl of the supernatants tested. Samples treated with APCB-BSA contained 25 μg UDA (or 25 μg tobacco chitinase) and 40 μg APCB-BSA in a final volume of 100 μl. The mixtures were kept at 37°C for 1 hour and subsequently at 4°C for 48 hours. The precipitates were collected by centrifugation (10,000*g*, 10 min) and redissolved in 50 μl sodium phosphate buffer containing 1 m*M* chitotriose.

Treatment	UDA		Tobacco chitinase	
	Hyphal growth inhibition activity	Hemagglutination activity	Hyphal growth inhibition activity	Chitinase activity
Untreated	100	100	100	100
Chitotriose addition (10 m*M*)	36	0	20	57
Chitotriose addition (1 m*M*)	92	0	94	100
Immunoprecipitation with anti-UDA (supernatant)	15	12	100	98
Immunoprecipitation with anti-chitinase (supernatant)	94	95	0	5
Precipitation with APCB-BSA (precipitate)	75	80	0	0
Heating (10 min at 75°C)	100	100	0	0
Heating (10 min at 85°C)	15	12	0	0

ovomucoid-Sepharose column, caused only a 40% reduction of the growth of *B. cinerea* hyphae. This experiment indicated that UDA is a major proteinaceous antifungal agent in stinging nettle rhizomes. Furthermore, immunocytochemical localization studies have shown that UDA is distributed predominantly throughout the cortex of rhizomes and in the outer exodermis cell layer of roots, whereas the lectin is absent from stems and leaves of *Urtica dioica* plants (*20*). The occurrence of UDA at the periphery of the underground organs is consistent with a possible role in the defense against potentially pathogenic fungi.

Apical growth of fungal hyphae depends on a delicate balance between chitin synthesis and selective hydrolysis of preformed chitin chains (*21*). The suggestion of Mirelman *et al.* (*3*), that chitin-binding lectins can disturb this balanced growth by binding or crosslinking chitin chains, may also apply to UDA. However, the particular small size of this lectin may render it more accessible to the active sites involved in cell wall morphogenesis, and hence, more active in inhibiting hyphal growth than larger chitin-binding lectins such as WGA or potato lectin (molecular masses of 36 kD and 100 kD, respectively). UDA could prove promising for possible applications in the genetic engineering of disease-resistant plants because of its simplicity as a single gene product, its distinct antifungal properties, and its restricted distribution in the plant kingdom.

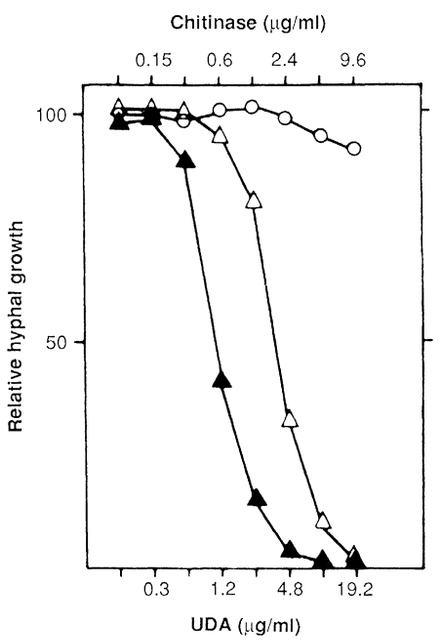

Fig. 3. Synergism between UDA and tobacco chitinase. Hyphal growth of *T. hamatum* was measured (as described in the legend to Fig. 1) at different concentrations of UDA (○), tobacco chitinase (△), and UDA and tobacco chitinase combined at a 2-to-1 mass ratio (▲). Relative hyphal growth is expressed as a percentage of the hyphal growth of control cultures; data are means of three independent experiments. Ratios of standard errors to means were 11% or less. Protein concentration is presented on a logarithmic scale.

REFERENCES AND NOTES

1. M. E. Etzler, *Annu. Rev. Plant Physiol.* **36**, 209 (1985).
2. H. Rudiger, *Bio Sci.* **34**, 95 (1984).
3. D. Mirelman *et al.*, *Nature* **256**, 414 (1975).
4. M. Mishkind *et al.*, *J. Cell Biol.* **92**, 753 (1982).
5. R. Barkai-Golan, D. Mirelman, N. Sharon, *Archs. Microbiol.* **116**, 119 (1978).
6. R. Brambl and W. Gade, *Physiol. Plant.* **64**, 402 (1985).
7. A. Schlumbaum *et al.*, *Nature* **324**, 365 (1986).
8. W. J. Peumans, M. De Ley, W. F. Broekaert, *Fed. Exp. Biol. Soc. Lett.* **177**, 99 (1983).
9. N. Shibuya *et al.*, *Arch. Biochem. Biophys.* **249**, 215 (1986).
10. M. P. Chapot, W. J. Peumans, A. D. Strosberg, *Fed. Exp. Biol. Soc. Lett.* **195**, 231 (1986).
11. UDA was isolated from stinging nettle rhizomes as described by Peumans *et al.* (*8*). However, the lectin was further purifed by high-performance gel filtration on a Superose-12 column (Pharmacia), with phosphate buffered saline (PBS) as a running buffer. The lectin fraction was subsequently subjected to affinity chromatography on ovomucoid Sepharose. Washing buffer was PBS and desorbent 50 m*M* acetic acid. UDA was dialyzed against water, lyophilized, and stored at 4°C.
12. J. Molano *et al.*, *Anal. Biochem.* **83**, 648 (1977).
13. W. F. Broekaert, J. Van Parijs, A. K. Allen, W. J. Peumans, *Physiol. Mol. Plant Pathol.* **33**, 319 (1988).
14. J. S. Price and R. Storck, *J. Bacteriol.* **124**, 1574 (1975).
15. R. J. Cohen, *Plant Sci.* **43**, 93 (1986).
16. F. B. Abeles *et al.*, *Plant Physiol.* **47**, 129 (1970).
17. S. Bartnicki-Garcia and E. Lippman, in *Handbook of Microbiology*, A. I. Laskin and H. A. Lechevalier, Eds. (CRC Press, West Palm Beach, FL, ed. 2, 1978), vol. 4, pp. 87–252.
18. Isolation of WGA was done as described by W. J. Peumans, H. M. Stinissen, and A. R. Carlier [*Planta* **154**, 562 (1982)]. Potato lectin was purified as describing R. J. Owens, and D. H. Northcote [*Phytochemistry* **19**, 1861 (1980)].
19. J. Van Parijs, unpublished results.
20. D. Lambrechts and J.-P. Verbelen, unpublished results.
21. V. Farkas *Microbiol. Rev.* **43**, 117 (1979).
22. O. D. Dhingra and J. B. Sinclair, in *Basic Plant Pathology Methods* (CRC Press, Boca Raton, FL, 1985).
23. Culture of fungi and collection of spores was done according to published protocols [see Broekaert *et al.* (*13*) for *T. hamatum*, *T. viride*, *B. cinerea*, and *Phycomyces blakesleeanus*, and Dhingra and Sinclair (*22*) for *Phoma betae*, *C. lindemuthianum*, *Phytophthora erythroseptica*, and *S. nodorum*.

24. Supported in part by grants of the National Fund for Scientific Research (Belgium); W.J.P. is senior research associate, and W.F.B. research assistant of this fund; J.V.P. received a fellowship of the Belgian Instituut tot Aanmoediging van het Wetenschappelijk Onderzoek in Nijverheid en Landbouw. We thank A. Van Laere and J. Vanderleyden for discussion.

2 March 1989; accepted 6 June 1989

Was Adenine the First Purine?

ALAN W. SCHWARTZ AND C. G. BAKKER

Oligomerization of HCN (1 molar) in the presence of added formaldehyde (0.5 molar) produced an order of magnitude more 8-hydroxymethyladenine than adenine or any other biologically significant purine. This result suggests that on the prebiotic Earth, nucleoside analogs may have been synthesized directly in more complex mixtures of HCN with other aldehydes.

FORMALDEHYDE AND HCN ARE THE simplest and most plausible starting materials for prebiotic syntheses on the early Earth (*1–3*). Formaldehyde is expected to have been ubiquitous, because of the ease of its photochemical formation from CO_2 and water vapor, as well as its formation from methane and water vapor by electric discharge, and HCN is produced efficiently by electric discharge in nitrogen-methane mixtures. In dilute aqueous solution at *p*H 9.2, HCN oligomerizes to produce a complex range of products (*4, 5*). Formaldehyde has been shown to accelerate this oligomerization (*6*). Adenine and traces of uracil have been identified among the products under other conditions, such as acid hydrolysis, that hydrolyze more complex precursors (*7*). Investigation of unhydrolyzed oligomerization mixtures prepared from HCN in the absence of formaldehyde has revealed the presence of the adenine precursor adenine-8-carboxamide (*8*). In this report we show, however, that the purine formed most in oligomerizing solutions of HCN to which formaldehyde has been added is 8-hydroxymethyladenine (HMA), rather than adenine-8-carboxamide or adenine itself.

We synthesized HMA according to a known procedure (*9*) and determined its chromatographic behavior on two high-performance liquid chromatographic (HPLC) columns: Aminex A25 (Bio-Rad) in 0.1*M* sodium formate (*p*H 4.0) and Aminex A6 in 0.1*M* NH_3 containing 0.02*M* ammonium carbonate (*p*H 10.0). Both columns were operated at 60°C. A fresh solution of formaldehyde (1*M*), prepared by sublimation from paraformaldehyde, was mixed with an

Laboratory for Exobiology, Faculty of Science, University of Nijmegen Toernooiveld, 6525 ED Nijmegen, the Netherlands.