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Short Communications

Isolation and partial characterization of a phytotoxic glycoprotein from culture filtrates of *Rhynchosporium secalis*[1]

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Summary. Rhynchosporium secalis (Oud.) Davis produces a phytotoxic compound with a mol. wt of 275×10^3 which is able to induce chlorotic symptoms in both susceptible and resistant barley leaves. Collectively, the data suggest that the toxin is a glycoprotein. Mild base treatment, by β elimination, indicates that threonine and serine are involved in o-glycosidic linkages with the carbohydrate moiety. Sugar residues occur in the molecule in the ratio of mannose, rhammnose, galactose, glucosamine 13.6:1:1:1.
Key words. Rhynchosporium secalis; barley scald disease; phytotoxin; glycoprotein; phytotoxic; rhynchosporide.

Rhynchosporium secalis is the causal organism of scald disease in barley. A host-selective toxin called rhynchosporoside was isolated from culture filtrates of *R. secalis* and also from infected plants[2]. This toxin was able to reproduce scald symptoms with concentrations as low as 6–12 µg/ml[2]. The original proposed structure of rhynchosporoside was that of a cellobioside linked 2–0 α to 1,2 propanediol and this structure has been recently revised[3]. It turns out that *R. secalis* produces a toxic family of β 1–4 glucosides linked 1–0α to 1,2 propanediol, including the glucoside, the cellobioside, the cellotrioside[3,4] and the cellotetraoside[5]. The 3 lowest polymers of this family of glucosides have affinities for membraneous receptors both in vitro and in vivo[6,7]. The greater affinity is always displayed with the susceptible lines of barley. The compound of greatest affinity to the receptor was the cellobioside of 1,2 propanediol[7]. Preliminary biochemical data have pointed out that among these toxic glycosides the glucoside and the cellobioside have a strong, non-host specific, uncoupling effect on the chloroplastic electron transport, this effect occurring without prior binding to the thylakoids[8].

Thus, even with the great contribution of these toxins to the production of disease symptoms, some events like the overcoming of resistance during the natural process of infection cannot be attributed to the rhynchosporosides. In order to explain this phenomenon we examined the possibility that *R. secalis* produces other toxic molecules of higher molecular weight. Earlier workers claimed that some non-dialyzable compounds from culture filtrates of *R. secalis* were able to induce some of the scald symptoms on barley, oats and wheat[9]. Furthermore, other workers postulated the involvement of high molecular weight compounds, i.e. glycopeptides[4,10,11], in fungal infections of plants.

This paper reports the isolation and purification from culture filtrates of *R. secalis* of a phytotoxic polypeptide. In addition some preliminary data are provided concerning the structure and the biological activity of this phytotoxic compound.

Materials and methods. The seeds of near isogenic lines ATLAS 46 (resistant) and ATLAS (susceptible) were kindly provided by R. Eslick and H. Bockelman, Montana State University. They were grown in potting soil under greenhouse condi-

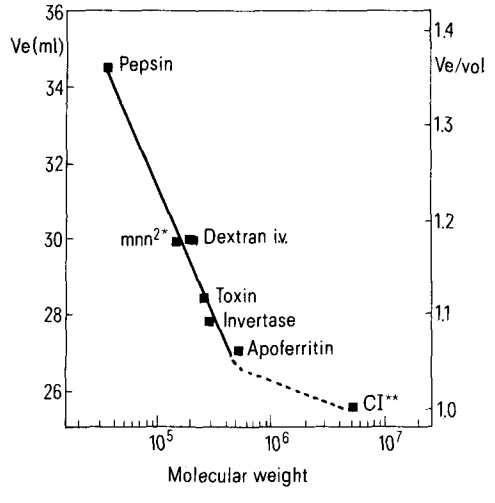
tions or in an environmentally controlled chamber at 13–21 °C night/day cycle with 12 h photoperiod. *R. secalis* used in this study was isolated from naturally infected barley leaves in Allier (France). The isolate provided by P. Pauvert INRA Versailles (France) was grown at 17 °C in 1 liter Roux bottles each containing 200 ml of the Fries No. 3 medium[12] with the following modifications: 7.5 g sucrose/l and yeast extract dialysate instead of crude yeast extract. Unless otherwise indicated all chemical products were from Sigma, St. Louis, Missouri, USA. The support Biogel A. 1.5 M was from Bio-Rad Laboratories, Richmond, California, USA.

Toxin Purification. Cultures of *R. secalis* were harvested after 13 days of incubation and filtered through a Miracloth disc (Calbiochem) on a filter funnel equipped with a coarse fitted disc (40–60) µm). The filtrate was concentrated to $\frac{1}{20}$ of the initial volume under reduced pressure at 50 °C. The concentration was precipitated with 2 volumes of cold acetone (−20 °C) and allowed to stand overnight at −20 °C. The precipitate was obtained after centrifugation for 10 min at 10,000 × g and dissolved in water. Traces of acetone were removed under reduced pressure. The solution was exhaustively dialyzed against distilled water. After dialysis the insoluble materials were removed by centrifugation and the resulting supernatant was separated on a 10 ml Sepharose ConA column previously equilibrated with acetate buffer 10 mM pH 6 complemented with 1 mM NaCl, $MgCl_2$ and $CaCl_2$. The separation of ConA positive material was identical to that previously described[10]. The carbohydrate content of the material obtained after exhaustive dialysis of the eluate of the Sepharose ConA column was estimated by the anthrone method[13]. The proteins were determined according to a dye-binding method[14] with the Biorad protein assay (Technical bulletin no. 1051, Biorad Laboratories, 1979). *Isolation of the toxic glycopeptide.* The ConA positive material was submitted to fractionation on polyacrylamide gels 7.5% with a constant current of 1.5 m A per gel[15]. The gels were stained with a freshly prepared solution of Coomassie Blue[16]. The glycoproteins were detected with thymol and sulfuric acid reagent[17]. The interface stacking gel, a small pore gel containing the glycoprotein was excised and ground, and the glycoprotein was allowed to diffuse into water for 48 h. After centrifugation for 10 min at 30,000 g, the supernatant was concentrated and repurified on the affinity column. The eluting material was used for biological and chemical tests after exhaustive dialysis.

Bioassay tests. An excised leaf method was used to test fungal extracts for the presence of biological activity. Leaves of 7–10-day-old plants were placed in test tubes with test materials. Under the following conditions; light 1 × 10^5 erg/cm^2-sec, temperature 22 °C ± 3 and time 4 days, a typical toxic reaction is the development of chlorosis on the leaf blade 4 days after the administration of the toxin preparation. Under these conditions, the lowest concentration of toxin (macromolecular weight compounds) able to induce slight chlorotic symptoms was 5 µg/ml. At high concentrations the expression of chlorosis is greater and symptoms develop sooner. The more highly-purified toxin preparations express greater toxicity (more chlorosis) and do it more quickly than the more crude preparations. Exact quantitation of the toxin effect on barley leaves could not be made using the bioassay test.

Chemical methods. For amino acid analysis, samples of lyophilized toxin (0.5–1.5 mg) were refluxed in 6 N HCl under vacuum for 18 h at 110 °C. The hydrolyzed toxin was dried over NaOH then dissolved in 200 mM sodium citrate pH 2.2 and analyzed according to Spackman et al[18]. Using a Beckman 120 C instrument. The nature of the carbohydrate-amino acid linkage was investigated by exposure of the toxin to mild base in order to incite β-elimination of threonyl and seryl o-glycosidic bonds[19]. The lyophilized toxin (0.5 mg) was treated according to[20] and dissolved in 200 µl of 0.1 N KOH or in 200 µl of 0.1 N KOH containing 0.1 N $Na_2(SO_3)$ at 37 °C for 20 h. The samples were neutralized with 1 N HCl and hydrolyzed by addition of an equal volume of 12 N HCl. Sugar analyses were performed with the techniques of Albersheim et al[21]. and Chambers et al.[22] using both the alditol acetate and the TMS methyl glycoside procedures, respectively.

Results. Biological activity. Initially, we decided to determine whether Con A positive material from *R. secalis* was deleterious to barley. Thus, this direct approach which was based on the experience of other workers permitted a rapid and effective method of toxin preparation. In combination with Con A affinity chromatography, gel electrophoresis was used to prepare the glycopeptide. The consolidated material from the final gel electrophoresis step (appearing at the top of the gel – termed purified toxin) at 90 µg/ml produced chlorotic symptoms in barley 1 day after treatment, whereas the crude acetone precipitated material (90 µg/ml) required more time for symptom expression suggesting that some purification had occurred. Absorbed quantities as low as 8–10 µg per leaf were able to in-



Molecular weight estimation of the toxin by gel filtration on a BIOGEL A 1.5 M (0.5 × 97 cm); *mnn^2, Mannoprotein from *Saccharomyces cerevisiae* × 2180-1A5 mnn^2 mutant[19]; **CI, Toxin from *Corynebacterium insidiosum* with a mol. wt of 5.10^6 [23] used to determine the void volume of the column.

Table 1. Amino acid analysis of the glycoprotein toxin produced by *R. Secalis*

Amino acid	nmoles[a]	Weight %[c]
Lysine	24.7	4.6
Histidine	11.3	2.1
Arginine	13.4	3.0
Aspartic	73.5	12.2
Thréonine[b]	99.0	14.5
Serine[b]	100.0	12.6
Glutamic	60.0	11.2
Proline	29.5	4.1
Glycine	54.4	4.5
Alanine	70.6	7.3
Cystine	trace	–
Valine	35.4	5.1
Méthionine	4.4	0.8
Isoleucine	24.4	4.0
Leucine	31.2	5.1
Tyrosine	13.6	3.2
Phénylalanine	15.1	3.3
Glucosamine[b]	8.8	2.3
		99.9

[a] Average of 2 determinations; [b] Not corrected for hydrolytic destruction; [c] Weight percentage of amino acids present. Total recovery of amino acids from hydrolyzed sample was 20.2 g per 100 g sample.

duce such symptoms whereas glucans at comparable concentrations, with a mol. wt of 2×10^5, were not toxic. Furthermore, the purified toxin caused a stabilized chlorotic lesion in 7 days on excised barley leaves when 10 µg of toxin per 10 µl H_2O was placed on a needle puncture wound. Controls having H_2O alone had no reaction. Glycans, at the same concentrations, were also not effective. In all cases of the bioassay tests, the resistent barley line Atlas 46 was more sensitive to the toxin than Atlas, the susceptible cultivar. Boiling for 15 min did not destroy the activity of the toxin.

Toxin production. The yield of toxin is low when compared to similar glycopeptides from other pathogenic fungi[10,23]. The average amount after 13 days of incubation is 0.8 mg/liter of culture filtrate. This represents about 3% of the total carbohydrate content of the precipitate (after acetone precipitation). After gel electrophoretic analysis of the Con A positive material, 7 bands were detectable with the Coomassie blue stain. One band stained intensively with the thymol-sulfuric acid reagent and it was located at the top of the small pore gel. It accounted for the majority of the carbohydrate containing macromolecules that were Con A positive. It possessed greater specific biological activity than the other bands on the gel and was thus used for further study and analysis (toxic glycopeptide).

Properties of the glycopeptide. Several compounds were used in the estimation of the molecular weight of the toxin. Among them were 3 glycoproteins, a toxin from *Corynebacterium insidiosum* of 5.10^6 mol. wt used to determine the void volume of the column, a mannoprotein (mnn 2) from a *Saccharomyces cerevisise* mutant[24] and invertase. All of these compounds as well as other proteins and the toxin were individually chromatographed on a 0.5 × 97 cm column of Bio-gel A 1.5 m and eluted with 10 mM acetate buffer pH 6.0 with a flow rate of 3.5 ml/l (fig.). The apparent mol. wt of the toxin determined after 2 experiments was 275×10^3. This estimate was in excellent agreement with the value of 300×10^3 determined using a Sepharose 4-B support as column packing material. The toxin gave a 280/260 absorption of 0.78 which is typical for glycoproteins[25].

Chemical Analysis of the Toxin. The toxin isolated from the cultures of *Rhynchosporium secalis* gave, after acid hydrolysis, a pattern of amino acids commonly encountered in proteins (table I). However, we noted a large amount of dicarboxylic amino acids, i.e. aspartic acid and glutamic acid which, with threonine and serine, accounted for 50% of the total weight of amino acids present. This must contribute to the strongly hydrophilic character of the toxin. Furthermore, the protein content of the toxin calculated from amino acid analysis is about 20.2%. This value is in good agreement with the value of 17.2% found by direct estimation with the Biorad method on the entire toxin.

Amino acid – sugar linkages. The presence of large amounts of threonine and serine in the toxin suggested their possible involvement in the amino acid-sugar linkages. Indeed, threonine and serine residues are selectively destroyed after treatment of the toxin with mild KOH. About half (58%) of the serine and threonine was lost as a result of the base treatment, and the 2 compounds are destroyed to an equal degree; this strongly suggested that the 2 amino acids participate to an equal extent in o-glycosidic linkages with the sugar of the carbohydrate moiety of the toxin (table 2). Sulfite addition in mild basic conditions showed that 80% of the threonine and serine were lost, which suggests an even greater involvement of these amino acids in o-glycosidic linkages. Considering the sulfonyl derivatives, i.e. cysteic acid and 2-amino-3-sulfonybutyric acid with the unmodified serine and threonine, they together account for 75% of the serine and threonine content of the non-treated toxin.

Sugar residue analyses revealed that mannose was the dominant residue in the glycopeptide (table 3). Rhamnose, galactose and glucosamine make up the majority of the remaining sugar residues. The total sugars present account for approximately 52.0% of the total weight of the glycopeptide. Note that the figures for the glucosamine by amino acid analyses are in close agreement with those obtained by sugar analyses (tables 1 and 2). High resolution proton NMR of the glycopeptide indicates that the mannosyl linkages are in the α-configuration[10]. By chemical analysis 72% of the glycoprotein can be accounted for in sugar or amino acid residues. The apparent loss of 28% of the compound after chemical analysis, could result in part from a possible hydratation of this very hydrophilic compound, leading us to overestimate the weight.

Discussion. Glycopeptides and other macromolecules are produced by a wide range of phytopathogenic fungi and bacteria[4]. These compounds may act to physically disrupt water flow within plant tissue[4]. Others may have membrane disruptive abilities[26]. The toxic macromolecule reported in this paper seems to be related to at least one other toxic fungal glycopeptide, i.e. *C. ulmi* glycopeptide in that both are mannorhamnanpeptides[10] with a mol. wt greater than 1×10^5.

At this point we cannot be sure that the *R. secalis* glycopeptide has any role in scald disease. However, the glycopeptide does produce most of the major symptoms of scald disease including chlorosis and marginal necrosis when artificially administered to the plant. It is the largest toxic compound found to date produced by *R. secalis*, which illustrates the extreme diversity of pathological tools in the arsenal of this parasite. Knowledge of the chemical and biological properties of the glycopeptide will dramatically aid efforts in understanding its role in the disease. For instance, the *C. ulmi* mannorhamnanpeptide acts as an antigen, which has permitted its rapid and easy detection in diseased plants[27]. The *R. secalis* glycopeptide does not represent a totally novel toxic compound among the group of toxins actually known, as far as the studies shown in this report have revealed. However it is enriched in threonine and serine which act as O-bridges to the glycosidic portion of the molecule (table 2).

Since it acts equally well, if not better, on the normal resistant host (ATLAS 46) as on the susceptible host (ATLAS), it would appear that the glycopeptide has virtually no role in dictating the specificity of the fungus for its host plant. Therefore fur-

Table 2. Effect of mild base treatment on the amino acid content of the toxin of *R. secalis*

Amino acid	Control[a]	KOH	KOH + $Na_2(SO_3)$
Aspartic[b]	1.00	1.0	1.0
Threonine	1.35	0.58	0.27
Serine	1.36	0.59	0.27
Cysteic[c]	0	0	1.5
Alanine	0.96	0.95	0.95

[a] Taken from table 1; [b] Aspartic has been assigned a value of 1.0, others amino acids are expressed as mole per mole aspartic; [c] The cysteic acid peak includes 2-amino-3-sulfonylbutyric acid.

Table 3. Weight percent of sugars detected in *R. Secalis* glycoprotein

	TMS	Alditol acetates	Average of alditol acetates and TMS
Rhamnose	2.0	4.0	3.0
Arabinose	–	0.4	0.2
Mannose	31.0	52.0	41.0
Galactose	3.0	4.0	3.0
Glucose	–	0.6	0.3
Glucosamine	6.0	0.7	3.0
Glactosamine	0	0	0
% of sample accounted for as carbohydrate	42.0	62.0	52.0

Fetuin was used as standard in order to check the 2 methods employed.

ther data on its molecular and chemical properties will be necessary for the understanding of its possible role in the overcoming of the host plant resistance.

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Evidence for the presence of arsenobetaine as a major arsenic compound in the shrimp *Sergestes lucens*[1]

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Summary. The major arsenic compound in the shrimp *Sergestes lucens* was isolated and identified as arsenobetaine $(CH_3)_3A_s^+CH_2COO^-$. Arsenobetaine accounted for 80% of the total arsenic in the shrimp.
Key words. Shrimp; arsenobetaine; arsenic; marine ecosystem; marine food.

It is well known that marine organisms contain more appreciable amounts of arsenic than terrestrial organisms[2]. From the viewpoint of food hygiene, this fact evokes a serious problem for those people who consume a lot of marine organisms as food. Since the toxic effects of arsenic depend on its chemical form, recent studies concerning the arsenic in marine organisms have concentrated on the elucidation of this; the compounds which have so far been clearly identified are arsenobetaine in some animals[3–8] and peculiar arsenosugars in the brown kelp[9] and the giant clam[10]. Among crustaceans 2 species of lobsters, western rock lobster *Panulirus longipes cygnus*[3] and American lobster *Homarus americanus*[6], have been shown to possess arsenobetaine. In the shrimp *Sergestes lucens,* which is a species closely related to the lobsters, the presence of an arsenobetaine-containing oligopeptide was recently suggested, but it was not fully purified[11]. This suggestion led us to isolate and identify the major arsenic compound in this shrimp. As described below, the results revealed that the major arsenic compound in the shrimp was not an arsenobetaine-containing oligopeptide but nothing other than arsenobetaine.

For the estimation of total arsenic, samples were digested with a mixture of nitric acid, perchloric acid and sulfuric acid, dissolved in an appropriate volume of water, and applied to a Jarrell Ash inductively coupled plasma emission spectrometer (AtomComp Series 800). In the course of purification, arsenic was determined on the same spectrometer without wet-digestion.

Fresh specimens of *S. lucens* (1.2 kg, As 5.5 ppm) obtained at Tokyo Central Wholesale Market were lyophilized and ground to powder. The powdered sample was extracted 3 times with methanol and the methanolic extracts were concentrated to dryness. The residue obtained was suspended in a small volume of water and defatted 3 times with an equal volume of ether. The aqueous phase (water-soluble fraction) which contained approximately 90% of the total arsenic in the starting specimens was used for the following purification procedure. The water-soluble fraction was first subjected to adsorption chromatography on Amberlite XAD-2. The unadsorbed fraction was applied to a column of Dowex 50 × 2 (H^+ form) equilibrated with water. The major arsenic compound was adsorbed by the column and could be eluted with 0.2 N ammonium hydroxide after washing the column with water. The eluate containing arsenic was combined, concentrated to remove ammonia, and passed successively through columns of Dowex 2 × 8 (OH^- form) and Amberlite CG-50 (H^+ form) both of which were previously equilibrated with water. The arsenic fraction was then put onto a column of Dowex 50 × 2 (pyridinium form) which was equilibrated with 0.1 M pyridine-formic acid buffer (pH 3.1). When eluted with the same buffer, the arsenic compound exhibited a weak interaction with the column and was eluted in volumes between 3 and 4 times the column volume. Chromatographic separations on Amberlite CG-50 (H^+ form) and Dowex 50 × 2 (pyridinium form) were performed again. Finally, gel filtration on Bio-Gel P-2 with water yielded 5.7 mg of the purified arsenic compound. The arsenic content of the purified compound was 40% which is comparable to the calculated value for arsenobetaine (41%). When analyzed by TLC on precoated silica gel 60 plates