The structures of the honeydew oligosaccharides synthesized by *Claviceps africana*

Amauri Bogo¹, Peter G. Mantle², Ricardo T. Casa¹, Altamir F. Guidolin¹

¹Departamento de Fitotecnia, Centro de Ciências Agroveterinárias, CAV, Universidade do Estado de Santa Catarina, UDESC, Av. Luís de Camões, 2090, CP 281, 88520-000, Lages/SC; ²Department of Biochemistry, Imperial College of Science, Technology and Medicine, London SW7 2AY United Kingdom.

Autor para correspondência: Amauri Bogo. <a2ab@cav.udesc.br>

Data de chegada: 31/05/04. Aceito para publicação: 24/03/05.

ABSTRACT

The structures of the principal oligosaccharides in the honeydew exudate of the sorghum ergot pathogen *Claviceps africana*, which has become epidemic in the Americas, have been characterized through linkage analysis using FAB-MS and GC-MS techniques, as 1,6-di-b-D-fructofuranosyl-D-mannitol and 1,5-di-b-D-fructofuranosyl-D-arabitol trisaccharides, 1-b-D-fructofuranosyl-D-mannitol and 5-b-D-fructofuranosyl-D-arabitol disaccharides and other minor disaccharides and trisaccharides. Their structural diversity is explained according to perceived biosynthetic interrelationships in pathways that appear to be unique amongst ergot fungi, particularly concerning intra-molecular reduction of fructose. The oligosaccharide, 1,6-di-b-D-fructofuranosyl-D-mannitol, which inhibits *C. africana* macrospore germination at a concentration in water of 1 g/mL or more, forms together with other slightly less bioactive oligosaccharides, the basis of a novel strategy to limit ergot disease losses in hybrid sorghum seed production.

Additional key words: ergot, linkage analysis, FAB-MS, GC-MS.

RESUMO


As estruturas dos principais oligossacarídeos presentes no exsudato da doença açucarada do sorgo, produzidos pelo patógeno *Claviceps africana*, foram caracterizadas através de espectroscopia de bombardeamento rápido de átomos-espectrometria de massa (FAB-MS) e cromatografia gasosa-espectrometria de massa (GC-MS). Dois grupos de oligossacarídeos foram elucidados, sendo o principal formado pelos trissacarídeos 1,6-di-b-D-frutofuranosil-D-mannitol e 1,5-di-b-D-frutofuranosil-D-arabitol e os dissacarídeos 1-b-D-frutofuranosil-D-mannitol e 5-b-D-frutofuranosil-D-arabitol e outros grupos menos importantes de dissacarídeos e trissacarídeos. As diversidades estruturais foram elucidadas de acordo com a inter-relação biosintética de sua rota metabólica, a qual parece ser exclusiva entre os fungos produtores da doença “ergot”, particularmente com relação a redução intramolecular da frutose. O oligossacarídeo 1,6-di-b-D-frutofuranosil-D-mannitol, o qual inibe a germinação dos macrosporos de *C. africana* a uma concentração de 1 g/mL ou mais, forma juntamente com outros oligossacarídeos menos bioativos a base de uma nova estratégia para limitar as perdas na produção de sementes de híbridos de sorgo.

Palavras-chave adicionais: ergot, análise de ligação, doença açucarada do sorgo.

The sugar composition of the honeydew exudate from ergot fungi parasitising host grasses and cereals has, until recently, been largely of academic interest, concerning only the presumed mixture of oligosaccharides arising from the pathogen’s preferred use of the glucose component of the host’s sucrose (14). However, a prominent feature of the sorghum ergot pathogen *Claviceps africana* Frederickson, Mantle & De Milliano (8), indigenous to parts of Africa, is the development of a white crust of secondary spores on the surface of exuded honeydew (7), from which the name “sugary disease” arises. Unlike other important ergot pathogens, the asexually produced secondary spores of *C. africana* are potentially windborne (9), which makes them an important epide-
Following the discovery of this disease in the Americas in 1995, secondary sporulation facilitated the dramatic spread of the pathogen in sorghum within South America in 1996 and prompted the first unequivocal recognition of the pathogen’s identity (15). The disease spread through Central America (16) into the USA in 1997 (11), where the fungus now is a serious economic threat to commercial hybrid sorghum seed production. *C. africana* occurs in Australia, Japan and Thailand and has also recently been recognised in India (2). Recognition of the potential global epidemiological significance of the secondary sporulation phenomenon (12) of this pathogen has focused attention to the sugar composition of the honeydew exudate in allowing or facilitating production of airborne inoculum (10, 3). The present saccharides studies have particularly addressed the report on the occurrence of oligosaccharides in the honeydew of *C. purpurea* (Fr.) Tul. (5). *C. africana* has alditol components and these were quite different from the honeydew sugars of *C. purpurea* which the biosynthesis is different from those ergot fungi pathogens (3).

The present paper reports on a fresh evaluation of *C. africana* oligosaccharides, in the context of the new global significance of sorghum ergot disease, primarily applying methodology (FAB-MS and GC-MS in electron impact and chemical ionisation modes) to define new oligosaccharides components. It is also reported that the main oligosaccharides are inhibitors to *C. africana* macrospore germination at quite low concentration, thereby offering a novel strategy to limit epidemics of ergot disease in hybrid sorghum seed production through the use of female lines expressing a sweet character.

**MATERIAL AND METHODS**

**Ergot parasitism.** Male-sterile sorghum (*Sorghum bicolor* L.) (IS 2219 A) was grown in a horticultural tunnel at the Chelsea Physic Garden, London, and flowered in August and September. Florets were inoculated with *C. africana* at floret gaping by spraying diluted natural honeydew with spore concentration around 1x10⁴ conidia/mL. New parasitized tissues exuded honeydew 1-2 weeks after floret inoculation.

**Oligosaccharides isolation.** Descending paper chromatography [Whatman 3MM No. 1 paper; solvent, propan-1-ol:ethyl acetate:water (7:1:2)] for 48-55 h resolved standards (D-fructose, D-glucose, sucrose and raffinose) and analytically and/or preparatively separated sugars in 100 ml of honeydew. To localise the sugars, lateral strips of the chromatograms were stained in aniline hydrogen phthalate reagent (3) and heated at 120°C for 20 min. The oligosaccharides separated in the chromatograms were eluted though a 2.5 x 40 cm column of Bio-Gel P-2 (Bio-Rad) equilibrated in degassed water at 0.4 mL/min. The eluate was monitored with a Waters RI-detector R-403 and the fractions (5 mL) were collected with an LKB fraction collector, using an Hitachi 561 Recorder. The fractions collected were freeze-dried prior to saccharide analysis and bioassay.

**Linkage analysis.** Oligosaccharide composition was determined by a combination of FAB-MS and GC-MS analysis. Oligosaccharides were reduced on a DuPont Model 21-491 instrument with dry DMSO plus 5 pellets of NaOH, reduced with 200 mL of borohydride and permethylated with 0.5 mL methyl iodide, as described by Dell et al. (6), before FAB-MS analyses. GC-MS analysis after the standard procedure of hydrolysis in 1M trifluoroacetic acid (TFA) at 25°C for 30 min, followed by reduction with sodium borodeuteride and acetylation with acetic anhydride had been applied to the permethylated saccharides, as described by Carpita & Shea (4). GC-MS was carried out on a Finnigan/MAT 9610 gas chromatograph equipped with a 30 m x 0.2 mm DB-5 capillary column in a temperature gradient 90-190°C (20°C min⁻¹), 190-210°C (1°C min⁻¹), 210-300°C (25°C min⁻¹). In A a Fisons 8000 quadrupole mass spectrometer system in electron impact mode, and B employed a VG autospek Q system in chemical ionisation with negative ion mode to show molecular ions of derivatised monosaccharides.

**Macroscope germination.** Potato dextrose agar (Difco) was diluted to 1/8 normal concentration and supplemented with agar (2%). Purified honeydew oligosaccharides, or raffinose, manitol or arabinol as controls, were incorporated into the medium at concentrations up to 5% and a drop spread as a 1 cm diameter film on a microspore slide. The surface was inoculated by sedimenting in the air an aerosol of parasitically-produced conidia in water. Slides were incubated in a water-saturated atmosphere in Petri dishes at 18°C. Without added oligosaccharides, macrospores germinated by an iterative process (8), giving a secondary spore on a sterigma projecting above the medium surface within 1-2 days. Spore germination was assessed microscopically.

**RESULTS**

Apparently, FAB-MS spectra of *C. africana* honeydew indicated the presence of two groups of oligosaccharides, one with ions around m/z 450-500 and the other around m/z 650-700 (Figure 1). The first one seems to be a disaccharide group with ions m/z 449, 477 and 493, which are the respective pseudomolecular ion fragments of the permethylated sodiated disaccharide [M + Na]⁺. The most prominent FAB-MS disaccharides, eluted from paper chromatograms, are the ions m/z 449 and 493 (Figure 1, A and C). Ion m/z 449 is consistent with an oligosaccharide composed of one permethylated hexose plus one permethylated pentose (C₁₉H₃₈O₁₀), an A-type fragment ion corresponding to the mass of the free reducing end terminal group and the mass of the cationising ion (Na²⁻), i.e. (1 x 204) + (1 x 191) + 31 + 23. The ion m/z 477 (Figure 1, B) equate to di-hexose and can apply to several disaccharides including inulin, levansucrose and/or others which are composed of two permethylated hexoses, an A-type fragment ion corresponding to the mass of the non-reducing and free reducing end terminal groups and the mass of the cationising ion, i.e. (2 x 204) + 15 + 31 + 23. The data from GC-MS linkage analysis, of the ion m/z 477, showed small amounts of fructoses linked in positions 1 or 6 (Table 1) which, when each is associated with 2-fructose from this dominant component, reveals the presence of both levansucrose and inulobiose. The ion m/z 493 (Figure 1, C) by analogy shows an oligosaccharide composed of two permethylated hexoses, one of which was reduced, an A-type fragment ion of which corresponds to the mass of the non-reducing end terminal group, an additional increment of the reduced-reducing end and the mass of the cationising ion, i.e. (2 x 204) + 15 + 47 + 23. GC-EIMS and GC-CIMS data of the electron impact mode and chemical ionisation of the 1-b-D-fructofuranosyl-D-mannitol (Table 1) confirmed linkage of the fructofuranosyl component by the 2-position and is consistent with the mannitol being...
linked by either its 1- or 6- position.

A higher mass group of ions, m/z 653, 681 and 697 (Figure 1, D, E and F) are the respective sodiated pseudomolecular ions [M + Na]+ of the reduced trisaccharides. The most prominent FAB-MS trisaccharides, eluted from paper chromatograms, are represented by the ions m/z 653 and 697 (Figure 1, D and F). The ion m/z 697 correspond to [M + Na]+ for a molecular weight of 674, which is 47 amu in excess for a permethylated tri-hexose, showing the reduction of one hexose. This ion m/z 697 represents, together with the data from the GC-MS relative intensities of mass spectra fragment ions (Table 1), the major trisaccharide 1,6-di-b-D-fructofuranosyl-D-mannitol (two permethylated hexoses plus permethylated mannitol) which is composed of three permethylated hexoses, an A-type fragment ion corresponding to the mass of the non-reducing end terminal group, an additional increment of the reduced-reducing end and mass of the cationising ion, i.e. (3 x 204) + 15 + 47 + 23, gives the observed ion m/z 697. GC-EIMS data after the standard derivatisation procedure for linkage analysis implied that this trisaccharide contained an alditol linked at positions 1 and 6 and also the same fructofuranose as occurs in sucrose, linked at C3. Data from GC-CIMS complemented the limited fragment ion data of EIMS and confirmed particularly the molecular mass of derivatives of 1, 6-linked mannitol and 2-linked fructofuranose (Table 1).

FAB-MS data, after permethylation, showed others trisaccharides sodiated with ions m/z 653 and 681 (Figure 1, D and E) which equate to 204 amu (hexose) additions to the sodiated molecular ions of the disaccharides m/z 449 and 477, respectively (Figure 1, A and B). This data, together with that of linkage analysis, of each pure compound, showing a 1,6 linked fructose (Table 1), confirms occurrence of 1,5-di-b-D-fructofuranosyl-D-arabitol but also reveals a novel tri-fructose composed of 2-1 and 2-6 linked moieties, termed “inulevan-triose”.

Figure 1 – Fast atom bombardment-mass spectrum of permethylated oligosaccharides in natural honeydew of *Claviceps africana*. A) fructosyl-arabitol, B) di-hexoses, C) fructosyl-mannitol, D) difructosyl-arabitol, E) tri-hexoses, and F) difructosyl-mannitol. Numbers relate to the mass/charge value (m/z) of the pseudo molecular ion [M+Na]+ of each oligosaccharides with identical mass.

Table 1. Gas chromatography retention time of derivatives of purified monosaccharides, which were either free or were released during linkage analysis of oligosaccharides from the honeydew of *Claviceps africana*, together with the relative intensities of mass spectral fragment ions seen in electron impact mode, correlated with molecular ions seen in chemical ionisation mode.

<table>
<thead>
<tr>
<th>Derivatised monosaccharides</th>
<th>Important fragment ions (GC-EIMS)</th>
<th>GC-CIMS</th>
<th>LINKAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2,3,4,5-penta-O-methylpentitol</td>
<td>11.57</td>
<td>250</td>
<td>D-arabitol</td>
</tr>
<tr>
<td>1-O-acetyl-1,2,3,4,5-tetra-O-methylpentitol</td>
<td>14.18</td>
<td>251</td>
<td>5 - arabinol</td>
</tr>
<tr>
<td>1,2,3,4,5,6-hexa-O-methylhexitol</td>
<td>14.70</td>
<td>294</td>
<td>D-mannitol</td>
</tr>
<tr>
<td>1,5-di-O-acetyl-1,2,3,4-tri-O-methylpentitol</td>
<td>16.60</td>
<td>279</td>
<td>1,5 - arabinol</td>
</tr>
<tr>
<td>6-O-acetyl-1,2,3,4,5-penta-O-methylhexitol</td>
<td>17.03</td>
<td>295</td>
<td>5 - mannitol</td>
</tr>
<tr>
<td>5-O-acetyl-1,2,3,4,5-penta-O-methylhexitol</td>
<td>17.92</td>
<td>295</td>
<td>D-fructose</td>
</tr>
<tr>
<td>2,5-di-O-acetyl(2D)-1,3,4,6-tetra-O-methylhexitol</td>
<td>17.95</td>
<td>324</td>
<td>2 - fructose</td>
</tr>
<tr>
<td>1,5-di-O-acetyl(1D) 2,3,4,6 tetra-O-methylhexitol</td>
<td>18.64</td>
<td>295</td>
<td>1 - glucose</td>
</tr>
<tr>
<td>5-O-acetyl-1,2,3,4,6 (1D)-penta-O-methylhexitol</td>
<td>18.67</td>
<td>295</td>
<td>D-glucose</td>
</tr>
<tr>
<td>1,6-di-O-acetyl-1,2,3,4,5 tetra-O-methylhexitol</td>
<td>19.15</td>
<td>323</td>
<td>1,6 - mannitol</td>
</tr>
<tr>
<td>1,2,5-tri-O-acetyl(2D) 3,4,6 tri-O-methylhexitol</td>
<td>19.77</td>
<td>19.77</td>
<td>1 - fructose</td>
</tr>
<tr>
<td>2,5,6-tri-O-acetyl(2D) 1,3,4 tri-O-methylhexitol</td>
<td>19.77</td>
<td>19.77</td>
<td>6 - fructose</td>
</tr>
<tr>
<td>1,2,5-tri-O-acetyl-2,3,4,6-tetra-O-methylhexitol</td>
<td>19.84</td>
<td>19.84</td>
<td>1,2 - fructose</td>
</tr>
<tr>
<td>1,2,5,6-tetra-O-acetyl(2D) 3,4 di-O-methyl hexitol</td>
<td>19.84</td>
<td>19.84</td>
<td>1,6 fructose</td>
</tr>
</tbody>
</table>

abbreviations: +++, well-defined in spectrum; ++, middle-defined in spectrum; +, weakly-defined in spectrum.
The second most prominent trisaccharide is the ion m/z 653 (Figure 1, D) which represent, together with the data from the GC-MS relative intensities of mass spectra fragment ions (Table 1) the 1,5-di-b-D-fructofuranosyl-D-arabitol (two permethylated hexoses plus permethylated arabitol). The minor trisaccharides have the ions m/z 681. The ion m/z 681 (three permethylated hexoses) covers a group including, theoretically, 1-kestose, 6-kestose, inulintriose or any other tri-hexose.

Concerning the oligosaccharides biological activities, the trisaccharide 1,6-di-b-D-fructofuranosyl-D-mannitol at 1 g/mL inhibited macrospore germination by 94% and at 2 g/mL, the spore germination was completely inhibited. The 1-b-D-fructofuranosyl-D-mannitol disaccharide was completely inhibitory at 5 g/mL but had no effect at 2 g/mL. The 1,5-di-b-D-fructofuranosyl-D-arabitol and 5-b-D-fructofuranosyl-D-arabitol also completely inhibited at 5 g/mL but were 70% inhibitory at 2 g/mL and 40% inhibitory at 1 g/mL. By contrast, pure compounds of raffinose, D-mannitol disaccharide was completely inhibitory at 5 g/mL but the 1-b-D-fructofuranosyl-D-arabitol dextrose inhibitory activity which it is to produce the alditol-containing oligosaccharides. Since secondary sporulation is only an in vitro phenomenon in C. sorghi (13), the anamorph of which (Sphaeria sorghi) is morphologically indistinguishable from that of C. africana, it is important not to be confused where C. africana is misnamed as C. sorghi (1). A reason why C. sorghi never significantly exhibited secondary sporulation in vivo concerned the less bulky growth habit of its sphacelium (9). This results in less demand on the host’s sucrose and a much higher saccharide concentration in honeydew. In this case, probably the high content of fructose is alone sufficient to account for the general failure to produce the white cascade of secondary sporulation on the surface of infected plants. However, it makes the point that it is the ratio between the pathogen’s demand and the host’s supply which most influences whether or not conidia in honeydew will germinate to release an airborne secondary spore. Even C. sorghicola, the anamorph of which is different from that of the other two Sorghum pathogens, can perform secondary sporulation in vivo (10), but this has never been reported as an in vivo event, again probably because of the dominance and abundance of fructose in the honeydew.

Normally, the developing sphacelium of C. africana, which is so bulky that it forces the glumes apart, utilises virtually all of the sucrose supplied by the grain Sorghum floret. This is why the concentration of saccharides in the honeydew is generally so low. The glucose moiety of sucrose is metabolised by the pathogen as a carbon and energy source, and the fructose moiety, partly reduced to mannitol, is incorporated into oligosaccharides. The efficient biosynthetic mechanism of linking mannitol to fructose molecules and the apparent abundance of the enzyme activity both fit and explain the increase in the concentration of bioactive oligosaccharides in honeydew where the sorghum plant has a ‘sweet’ character. The inevitability of perfusion of phloem leakage through a highly metabolic sphacelium ensures efficient utilisation of the sucrose, and transformation of excess in sweet sorghums to the oligosaccharides. The present mechanistic study therefore reinforces the idea that incorporation of a sweet character into A lines for sorghum hybrid production could provide an effective limitation on the adverse impact of ergot disease by restricting secondary sporulation on honeydew.

ACKNOWLEDGEMENTS

We acknowledge facilities for growth of male-sterile sorghum at the Chelsea Physic Garden, London and research studentship support from CAPES, Brazil. We thank J. Barton and R. Easton for obtaining GC-CIMS and FAB-MS data.
REFERENCES


