*Phytochemistry*, 1977, Vol. 16, pp. 1365–1368. Pergamon Press. Printed in England.

# PHYSICAL AND CHEMICAL PROPERTIES OF THE MUCIN SECRETED BY *DROSERA CAPENSIS*

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(*Revised received* 1 *April* 1977)

**Key Word Index**—*Drosera capensis*; *D. binata*; Droseraceae; mucin; acid polysaccharide; viscosity; molecular weight; carbohydrate and sulphate composition; gas–liquid chromatography.

**Abstract**—The mucin droplets secreted by the leaves of *Drosera capensis* consist of a 4% aq. solution of an acidic polysaccharide containing xylose, mannose, galactose, glucuronic acid and ester sulphate in the ratio 1:6:6:6:1. This polysaccharide is the only macromolecule in the secretion and is homogeneous on gel-filtration, ion-exchange chromatography, cellulose acetate electrophoresis and on ultracentrifugation ($S_{apparent}$ $4.4 \times 10^{-13}$). The MW is $> 2 \times 10^6$. The viscosity of the mucin in aq. solution decreases with changes in pH, increase in temperature, freezing and thawing or lyophilisation. The mucin contains 22 mM $Ca^{2+}$, 19 mM $Mg^{2+}$, 0.9 mM $K^+$ and 0.2 mM $Na^+$. Protein was absent from the secretion. The corresponding mucin from *Drosera binata* has similar properties and chemical composition.

## INTRODUCTION

Leaves of the carnivorous plants of the Droseraceae ('sundew') secrete droplets consisting of viscous mucin. Insects become trapped in the sticky fluid and are subsequently digested, probably by enzymes secreted from the leaves. Darwin [1] was the first to systematically study insectivorous plants including the *Drosera* species. In the following 100 years some chemical and enzymic properties of the secretions from different *Drosera* species have been investigated [2–6]. However, the chemical nature of the compounds responsible for the high viscosity of the secretion product has not yet been elucidated. By analogy to animal glycoproteins [7, 8] and proteoglycans [9, 10] and acidic polysaccharides such as mucilages and gums from plants [9–12], a highly polymerized glycoconjugate could be expected in sundew secretions. This assumption has been supported by recent cytochemical studies with *Drosera rotundifolia* and *D. capensis* [13]. In addition, the presence of a polysaccharide in the secretion from the related genus *Drosophyllum* has already been assumed by Meyer and Dewévre in 1894 [14] and has later been confirmed by Schnepf [15].

## RESULTS

### *Physical properties of* Drosera *mucin*

The sundew *Drosera capensis* produces a highly viscous mucin of pH 5 which can be obtained in good yield by gauze collection and represents an about 4% aq. solution of an acidic polysaccharide. The freshly secreted mucin is so sticky that it can be drawn into threads of about 1 m length. The mucin does not take up additional water and dissolves only very slowly in this solvent. It was not investigated whether the concentration of the fresh mucin is dependent on the humidity of the air.

The viscosity of a 0.2% solution was found to be six times higher than that of water at 20°. It is linearly proportional to the polysaccharide concentration up to 2 mg/ml. While maximum viscosity was observed at about pH 5, it irreversibly decreases when the pH is either raised or lowered. A rapid and irreversible decrease of the viscosity was also observed at higher temperatures. Raising the temperature from 0 to 80° leads rapidly to a tenfold decrease of the viscosity at nearly linear rate. While the viscosity of the native mucin is relatively stable at 2° it decreases slowly after dilution with water. In addition, the solution becomes turbid within a few days (bacterial growth was prevented by the addition of azide) and the sediment formed is insoluble. Freezing and thawing or lyophilisation also lead to a rapid loss of viscosity. The freeze-dried material is hygroscopic and has a gum-like consistency; it can only be very slowly dissolved in water.

The secreted mucin represents a pure solution of polysaccharide in water. Only minute amounts of coloured substances and monosaccharides were found in the concentrated diffusate of the colourless mucin solution.

The homogeneity of the polysaccharide was shown by the following physical and chemical investigations: the whole material is adsorbed on DEAE-cellulose and eluted almost as one peak by a NaCl-gradient. This experiment also demonstrates the acid nature of the mucin. On Sephadex G-200 the material was completely excluded. On Sepharose 4B, the mucin eluted as a single, however broad and slightly retained peak starting with the void volume. On Sepharose 2B the polysaccharide was completely retained and eluted as one peak. This behaviour of the polysaccharide in the gel-filtration experiments was not appreciably influenced by factors known to reduce the interactions of polysaccharide molecules in solution, such as alteration of the pH-value or of the salt concentration (0.2 M NaCl) or the addition of 6 M urea.

The polysaccharide also appeared homogeneous after electrophoresis in borate or veronal buffers on cellulose

acetate strips. In both buffers and at the pH-values between 4 and 12 the substance migrated (1.5–2 cm/30 min) as a single band towards the anode; At pH 3, mobility was reduced by 50%. This behaviour indicates the strong acidic nature of the mucin, the isoelectric point of which is assumed to be below pH 3. Due to these properties good staining of the mucin with toluidin blue and alcian blue was obtained. The spots could also be easily visualized with the periodic acid/fuchsin/sulphite reagent. They were not stained with amido black. Chromatography of the mucin on polyacrylamide or polyacrylamide–agarose gels was unsuccessful.

Homogeneity of the mucin polysaccharide was also demonstrated by ultracentrifugation. After sedimentation of a minute amount of particulate material from the freshly harvested, diluted mucin within 15 min at 9000 rpm a sharp peak appeared after 1 hr at 24000 rpm. It sedimented without alteration of its shape. The $S_{apparent}$ value of $4.4 \times 10^{-13}$ was calculated. No further peak appeared during centrifugation at 64000 rpm.

*Chemical properties of* Drosera *mucin*

Chemical and gravimetric methods demonstrated that the native secretion of the sundew leaves contains on an average 4% polysaccharide. Elementary analysis of the thoroughly dialysed mucin—partly against EDTA—has shown a composition by carbon, hydrogen and oxygen which is similar to that of polysaccharides. In addition, 1.2% of sulphur is present, and the content of nitrogen is negligible.

Analysis of the mucin and of its acid hydrolysate by colorimetry, TLC and GLC has demonstrated the presence of xylose, mannose, galactose and glucuronic acid in a molar ratio of 1:6:6:6. GLC of the TMSi derivatives of the methyl glycosides of the constituent monosaccharides from the mucin also showed the presence of a small, unidentified peak at 140° eluting before the known sugars. Analysis by GLC of the monosaccharides obtained by hydrolysis of the mucin in aq. acid gave analogous results. Sialic acids or other compounds leading to a chromophore in the periodic acid/thiobarbituric acid reaction [16] as e.g. 3-deoxy-octulosonic acid [17] could not be detected in the mucin after mild acid hydrolysis.

The polysaccharide contains 3% ester sulphate as calculated from elementary analysis and from turbidimetric sulphate determination. This corresponds to a molar ratio of about 1 when related to the ratio of the 4 monosaccharides given above.

2 mg of the polysaccharide de-cationized by previous dialysis against EDTA could be neutralized with $6 \times 10^{-6}$ mol NaOH. This value corresponds to the acid equivalents ($5 \times 10^{-6}$ mol) calculated from the sulphate and glucuronic acid content of a corresponding quantity of mucin. Most of the acid residues of the native mucin are neutralized by $Ca^{2+}$ and $Mg^{2+}$ ions. Concentrations of 22 mM $Ca^{2+}$, 19 mM $Mg^{2+}$, 0.9 mM $K^+$ and 0.2 mM $Na^+$ were determined in the native secretion.

Hexosamines or *N*-acetyl groups were not detected in the mucin; similarly, no indications for the presence of amino acids or peptide linkages were obtained by colorimetric tests. There was no influence of pronase on the physical properties of the mucin, e.g. depolymerisation which makes the presence of peptide sequences between the polysaccharide chains unlikely. Such depolymerisation reactions with pronase are known to occur with carbohydrate-rich glycoproteins or proteoglycans [18]. *O*-Acetyl groups or other ester linkages detectable by the hydroxylamine/$Fe^{3+}$ reagent do not occur in the polysaccharide. Protein analyses were similarly negative.

A mucin secreted by *Drosera binata* was also studied and found to be very similar to that of *D. capensis*.

## DISCUSSION

The sundew mucin is secreted as a relatively pure aq. solution of acidic polysaccharide of high MW. Its high viscosity suggests that the acidic polysaccharide molecules interact strongly and are highly hydrated [8, 19, 20]. The viscosity of the *Drosera* mucin decreases quickly under physical influences which are known to break the hydrogen bonds within and between the macromolecules. The viscosity of the sundew mucin decreases at higher temperatures which is in agreement with the behaviour of gums and of a microbial succinoglucan [21] but which differs from that of various mucins from animal [8] or bacterial [22] origin.

MW estimations using gel-electrophoresis, gel-filtration or ultracentrifugation of viscous solutions are hampered by the interactions of the macromolecules, e.g. as in heparin [23]. The physical studies described here mainly demonstrated the remarkable purity and homogeneity of the polysaccharide in the mucin. The sharp peak observed in ultracentrifugation is typical for viscous materials, especially for different types of acidic glycoconjugates and polysaccharides the molecules of which form aggregates [24–26]. From the behaviour of the substance on Sepharose 2B or 4B and the exclusion limit of $5 \times 10^6$ daltons for polysaccharides on Sepharose 4B a MW in the range of $2 \times 10^6$–$5 \times 10^6$ may be assumed. Further support for such a large MW was the observation that sodium dodecylsulphate, salts, and urea did not appreciably influence the behaviour of the polysaccharide during gel-filtration or during electrophoresis [27].

Chemical analyses of the polysaccharide made either in the native secretion or after chromatography on DEAE-cellulose or Sepharose led to identical results with regard to the nature and molar ratio of its components. In contrast to an earlier report of sialic acids in plants [28] the experiments presented here demonstrate its absence. In higher animals, however, mucous secretions are known to contain a relatively high amount of sialic acid [29].

Xylose and galactose have also been found in the polysaccharide secreted by *Drosophyllum* [15]. The occurrence of arabinose, rhamnose and gluconic acid in the *Drosophyllum* mucin, contrasts with our results on the *Drosera capensis* mucin. The presence of $Ca^{2+}$ and the negligible amounts of $Na^+$ and $K^+$ in the *Drosera capensis* mucin [cf. 30] corresponds with observations made with the *Drosophyllum* mucin [14].

## EXPERIMENTAL

*Preparation of mucin. Drosera capensis* was cultivated in insect free glass houses at an average temp. of 15° and 50% humidity. The mucin droplets were collected weekly between May and August using a coarse fibre asbestos cloth. The mucin harvest (5 g pooled droplets) was dissolved in 1 l. water and filtered through glass wool. The filtrate was dialysed for 20 hr against 3 l. $H_2O$ with 3 changes, followed by a 20 hr dialysis against 3 l. of a satd EDTA soln with 3 changes and another 20 hr dialysis against 3 l. $H_2O$ with 3 changes. The dialyses were carried out at 2°. The colourless contents of the dialysis bags were lyo-

philized or stored in the frozen state. The diffusates from the initial 20 hr dialysis against $H_2O$ were combined and lyophilized.

*Anion-exchange chromatography.* Mucin (3.5 mg) was dissolved in 1 ml of 1 mM Na borate buffer, pH 8.5, and applied to a DEAE-cellulose column equilibrated with the same buffer. The material was eluted with a linear gradient from 0–1 M NaCl. The eluate was monitored with the PhOH–$H_2SO_4$ reagent described below.

*Gel-filtration.* Glass columns were treated before use with an 1% soln of dimethyldichlorsilane in $C_6H_6$. Lyophilized mucin (5 mg) was dissolved in 1 ml 50 mM Tris/HCl 100 mM KCl buffer, pH 7.5, and 10 mg sucrose were added. The sample was passed through Sephadex G-200, Sepharose 4B or Sepharose 2B equilibrated with the Tris/KCl buffer. The eluate was monitored photometrically at 280 nm and with the PhOH–$H_2SO_4$ acid reagent.

*Electrophoresis.* Lyophilized mucin (10 μg) was applied to the middle of cellulose acetate strips (25 × 128 mm). Electrophoresis (200 V, about 2.5 mA per strip, 30 min, room temp.) was carried out at pH 3–12 using 150 mM borate/NaOH, borate/HCl or veronal/acetate buffers. The strips were stained with toluidine blue or alcian blue [31]. Disc-electrophoresis according to ref. [32] was carried out in 6 mm × 10 cm tubes in 7.5% or 3.75% polyacrylamide gels and in a gel consisting of 1.2% polyacrylamide and 0.6% agarose similar to refs. [27, 33]. Tris/glycine buffer (50 mM), pH 8.9, was used. Lyophilized mucin (50–200 μg) was applied to each tube. Some experiments were carried out in the Tris/glycine buffer containing 0.4% Na dodecylsulphate; the mucin samples were preincubated for 2 hr in 1% Na dodecylsulphate. The electrophoretic conditions were 60 V and 25 mA for 1 hr, followed by 90 V and 25 mA for 2 hr. Bromophenol blue served as a marker. The gels were stained with alcian blue or with the periodic acid/fuchsin/sulphite reagent [34].

*Analytical ultracentrifugation.* Mucin was diluted with the Tris/KCl buffer used for gel-filtration, immediately after harvest without further purification to give a soln of 0.6 mg dry material/ml. The soln was analysed in a Beckman ultracentrifuge model E, using the schlieren-optic method [35]. The speed of the AN-D or AN-H rotors increased from 8000 rpm to 32000 rpm within 3 hr and reached maximum 64000 rpm.

*Viscosimetry.* Mucin was diluted with different vols of $H_2O$ immediately after harvest without further purification. The viscosity of these solns was determined with $H_2O$ as reference at 20° using an Ostwald KPG-viscosimeter; the pH was varied between 1 and 11 by the addition of HCl or NaOH.

*Colorimetric methods.* Total carbohydrate content of the mucin or of isolated monosaccharide fractions after hydrolysis was determined with the PhOH–$H_2SO_4$ reagent [36], uronic acids according to ref. [37], hexosamines according to ref. [38], *N*-acetylhexosamines according to ref. [39], ester sulphate groups according to ref. [40], amido acetate groups according to ref. [41] ester acetate groups according to ref. [42], protein according to ref. [43] or with the micro-Biuret test [44] and amino acids with the ninhydrin reagent [45]. For all assays, appropriate reference compounds were used. The presence of sialic acids was checked according to refs [46] and [16], respectively.

*Cation determination.* Mucin was diluted with appropriate vols of deionized $H_2O$, and the concentration of $Na^+$, $K^+$, $Ca^{2+}$ and $Mg^{2+}$ was determined using a flame emission spectrophotometer and an atom absorption spectrophotometer, respectively.

*Hydrolysis of mucin.* Lyophilized material (2 mg) was dissolved in 1 ml of 4N HCl or 2N trifluoroacetic acid and hydrolysed for 4 hr at 100° [47, 48]. For protein analysis, the hydrolysis conditions were 6N HCl, 21 hr and 110°. The hydrolysates were freeze-dried. A mucin sample was also hydrolysed in 100 mM HCl, 80° and 1 hr for the release of acid sensitive substances with labile glycosidic linkage, as e.g. sialic acids [49]. The hydrolysate was then dialysed for 24 hr against the tenfold vol. of $H_2O$ with 3 changes and the diffusate was lyophilised. The dry residues were dissolved in appropriate vols of $H_2O$ and analysed by colorimetric methods or by combination of ion-exchange chromatography and TLC as follows.

For GLC 200 μg of the lyophilised mucin was hydrolysed for 24 hr at 90° in 1 ml N MeOH–HCl in sealed ampoules [50]. Thereafter, the mixtures were lyophilised.

*Ion-exchange chromatography.* The monosaccharide mixtures from aq. hydrolysis were fractionated into acidic, basic and neutral compounds by passage through Dowex 2 × 8 ($HCOO^-$-form) and then Dowex 50 ($H^+$-form). While the uncharged compounds were found in the combined neutral effluent and the $H_2O$ washings, the acidic compounds were eluted from Dowex 2 × 8 by 3 bed vols of N HCOOH and the basic compounds from Dowex 50 by the same vol. of N HCl. The samples were lyophilised before further analysis.

*TLC.* Monosaccharides were chromatographed on 0.3 mm cellulose thin-layers containing boric acid in various solvents [51]; the *n*-BuOH–Py–$H_2O$ (6 : 4 : 3) system was most frequently used. The plates were stained with alkaline $AgNO_3$ followed by spraying with thiosulphate.

*GLC.* The freeze-dried samples from methanolysis of 200 μg mucin were 12 hr treated at room temp. with 0.2 ml Py–$Ac_2O$ (1 : 1) [52]. After removal of the organic solvents in $N_2$ the samples were stored for 24 hr over KOH and $P_2O_5$ *in vacuo* before silylation [52]. GLC conditions: stationary phase, 120 cm glass column with 3 mm i.d. filled with 3% OV-17 on Gas Chrom Q, 100–120 mesh; gas flow rates, 75 ml/min for $N_2$, 75 ml/min for $H_2$ and 80 ml/min for air; temp. program, 80–280° with an increase of 7.5°/min; injection port, 280°. Identifications were carried out by standard procedures [53].

*Acknowledgements*—We thank Prof. Dr. M. H. Zenk, Ruhr-Universität Bochum, for his interest in this work and for support in cultivating the sundew plants. The cation determinations were carried out by Dr. M. Locher, Universität Tübingen. We also thank Mrs Margaret Wember for skilful technical assistance. This work was financially supported by the Deutsche Forschungsgemeinschaft (grants Scha 202/1 and 202/3).

## REFERENCES

1. Darwin, C. (1875) *Insectivorous Plants*. Murray, London.
2. Weber, F. (1938) *Protoplasma* **31**, 289.
3. Holter, H. and Linderstrøm-Lang, K. (1933) *Z. Physiol. Chem.* **214**, 223.
4. Amagase, S., Mori, M. and Nakayama, S. (1972) *J. Biochem.* **72**, 765.
5. Amagase, S. (1972) *J. Biochem.* **72**, 73.
6. Heslop-Harrison, Y. (1975) in *Lysosomes in Biology and Pathology* **4** (Dingle, J. T. and Dean, R. T. eds.), pp. 525–578. North Holland, Amsterdam.
7. Meyer, F. A., King, M. and Gelman, R. A. (1975) *Biochim. Biophys. Acta.* **392**, 223.
8. Snary, D., Allen, A. and Pain, R. H. (1973) *European J. Biochem.* **36**, 72.
9. Phelps, C. F. (1972) in *Oxford Biology Readers No. 27* (Head, J. J. and Lowenstein, O. E., eds.). Oxford University Press, Oxford.
10. Winterburn, P. J. (1974) in *Companion to Biochemistry, Selected Topics for Further Study* (Bull, A. T., Lagnado, J. R., Thomas, J. O. and Tipton, K. F., eds.) pp. 307–341. Longman, London.
11. Nelson, R. E. and Ander, P. (1972) *Carbohyd. Res.* **25**, 81.
12. Tomoda, M. and Nakatsuka, S. (1972) *Chem. Pharm. Bull.* **20**, 2491.
13. Dexheimer, J. (1976) *Cytobiologie* **13**, 307.
14. Meyer, A. and Dewèvre, A. (1894) *Bot. Ctrbl.* **60**, 33.
15. Schnepf, E. (1963) *Flora* **153**, 1.
16. Warren, L. (1959) *J. Biol. Chem.* **234**, 1971.
17. Charon, D. and Szabó, L. (1972) *European J. Biochem.* **29**, 184.
18. Kresse, H., Heidel, H. and Buddecke, E. (1971) *European J. Biochem.* **22**, 557.

19. Gottschalk, A. and Thomas, M. A. W. (1961) *Biochim. Biophys. Acta* **46**, 91.
20. Aspinall, G. O. (1970) *Polysaccharides*. Pergamon Press, Oxford.
21. Harada, T. and Yoshimura, T. (1965) *Agr. Biol. Chem.* **29**, 1027.
22. Maeda, I., Saito, H., Masada, M., Misaki, A. and Harada, T. (1967) *Agr. Biol. Chem.* **31**, 1184.
23. Skalka, M. (1965) *J. Chromatog.* **33**, 456.
24. Meyer, F. A., Preston, B. N. and Lowther, D. A. (1969) *Biochem. J.* **113**, 559.
25. Misaki, A., Saito, H., Ito, T. and Harada, T. (1969) *Biochemistry* **8**, 4645.
26. Gibbons, R. A. (1972) in *Glycoproteins, Their Composition, Structure and Function* (Gottschalk, A, ed.) 2nd edn. pp. 31–40. Elsevier, Amsterdam.
27. Peacock, A. C., Dingman, C. W. (1968) *Biochemistry* **7**, 668.
28. Onodera, K., Hirano, S. and Hayashi, H. (1966) *Agr. Biol. Chem.* **30**, 1170.
29. Gottschalk, A., Bhargava, A. S. and Murty, V. L. N. (1972) in *Glycoproteins, Their Composition, Structure and Function* (Gottschalk, A. ed.) 2nd edn. pp. 810–829. Elsevier, Amsterdam.
30. Anderson, D. M. W. and Stoddart, J. F. (1966) *Carbohyd. Res.* **2**, 104.
31. Rauen, H. M. (ed.) (1964) *Biochem. Taschenbuch*, Vol. 2, pp. 463–464. Springer, Berlin.
32. Maurer, H. R. (ed.) (1971) *Disc Electrophoresis*. Walter de Gruyter, Berlin.
33. Holden, K. G., Yim, N. C. F., Griggs, L. J. and Weisbach, J. A. (1971) *Biochemistry* **10**, 3105.
34. Caldwell, R. C. and Pigman, W. (1965) *Arch. Biochem. Biophys.* **110**, 91.
35. Lloyd, P. H. (ed.) (1974) *Optical Methods in Ultracentrifugation, Electrophoresis and Diffusion*. Claredon Press, Oxford.
36. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) *Anal. Chem.* **28**, 350.
37. Bitter, T. and Muir, H. M. (1962) *Anal. Biochem.* **4**, 330.
38. Johnson, A. R. (1971) *Anal. Biochem.* **44**, 628.
39. Reissig, J. L., Strominger, J. L. and Leloir, L. F. (1955) *J. Biol. Chem.* **217**, 959.
40. Dodgson, K. S. and Price, R. G. (1962) *Biochem. J.* **84**, 106.
41. Ludowieg, J. and Dorfman, A. (1960) *Biochim. Biophys. Acta* **38**, 212.
42. Hestrin, S. (1949) *J. Biol. Chem.* **180**, 249.
43. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
44. Bailey, L. (1967) *Techniques in Protein Chemistry*, p. 341. Elsevier, Amsterdam.
45. Moore, S. and Stein, W. H. (1954) *J. Biol. Chem.* **211**, 893.
46. Böhm, P., Dauber, S. and Baumeister, L. (1954) *Klin. Wochschr.* **32**, 289.
47. Marshall, R. D. and Neuberger, A. (1972) in *Glycoproteins, Their Composition, Structure and Function* (Gottschalk, A. ed.) 2nd edn. pp. 224–299. Elsevier, Amsterdam.
48. Adams, G. A. (1965) in *Methods in Carbohydrate Chemistry* (Whistler, R. L. and Wolfrom, M. L., eds.) Vol. V, pp. 269–276. Academic Press, New York.
49. Schauer, R. (1973) *Angew. Chem.* **12**, 127.
50. Bhatti, T., Chambers, R. E. and Clamp, J. R. (1970) *Biochim. Biophys. Acta* **222**, 339.
51. Hough, L. and Jones, J. K. N. (1962) in *Methods in Carbohydrate Chemistry* (Whistler, R. L. and Wolfrom, M. L. eds.) Vol. 1, pp. 21–31. Academic Press, New York.
52. Laine, R. A., Esselman, W. J. and Sweeley, C. C. (1972) *Methods Enzymol.* **28**, 159.
53. Leibnitz, E. and Struppe, H. G. (eds.) (1970) *Handbuch der Gas-Chromatographie*. Akad. Verlagsgesellschaft Geest & Portig, Leipzig.

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