Autolysis of Extracellular Glucans Produced *in vitro* by a Strain of *Claviceps fusiformis*

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SUMMARY

Claviceps fusiformis usually produces a stable viscous glucan during submerged culture fermentations. A new strain 139/2/1 G of the fungus, which subsequently autolysed this glucan to glucose, was studied and the autolysis ascribed to a constitutive $\beta 1 \rightarrow 3$ glucanase and a β -glucosidase which were detected as soon as the fungal hyphae differentiated to a sclerotial form. The glucanase production followed a sigmoid pattern, reaching a maximum within 12 days, and the liberated glucose contributed to renewed growth towards the end of the fermentation. A sucrase and a maltase were also detected. Maximum glucan autolysis was achieved by using a large spore inoculum. This maintained minimal viscosity throughout the fermentation and, by maintaining adequate aeration, has since facilitated ergot alkaloid production by the organism.

INTRODUCTION

Davis, Rhodes & Shulke (1965) described the significant decrease of extracellular glucan in submerged cultures of *Plectania occidentalis* and a *Helotium* sp. towards the end of the fermentation and they presumed that this was due to the secretion of an 'exocellular glucanase' by the organism. However, no autolysis was observed during studies on the structurally similar glucan produced by *Claviceps fusiformis* (Buck, Chen, Dickerson, & Chain, 1968). The production of alkaloids in submerged cultures by *C. fusiformis* is adversely affected by the formation of the viscous glucan, and during investigations on the alkaloid fermentation process a new variant strain (139/2/1 G) arose spontaneously and was selected for its ability to autolyse the extracellular glucan. The usual non-autolysing strains yielded a stable and highly viscous growth within 7 days, whereas the new variant autolysed completely after a further 7 to 10 days. In view of the structure of the extracellular glucan, its autolysis by strain 139/2/1 G has been investigated to determine whether this is due to the activity of glucanases similar to those reported by Reese & Mandels (1959) for a variety of fungi.

METHODS

Origin of the Claviceps fusiformis isolate used. The non-autolysing isolate designated 139/2/1 was obtained from *C. fusiformis* sclerotia collected in Senegal in November 1965 and kindly supplied by the Director, Centre de Recherches Agronomiques, Senegal.

Submerged culture procedures

Media. Cultures were maintained on agar (2% w/v) slopes of the following medium modified from Stoll *et al.* (1954): sucrose, 100 g.; L-asparagine, 10 g.; Ca (NO₃)₂·4H₂O, 1 g.; KH₂PO₄, 0.25 g.; MgSO₄·7H₂O, 0.25 g.; KCl, 0.125 g.; FeSO₄·7H₂O, 0.033 g.; ZnSO₄·7H₂O, 0.027 g.; L-cysteine hydrochloride, 0.01 g.; yeast extract, 0.1 g.; distilled water, 1 l.; pH, 5.2 adjusted with NaOH. For liquid culture 100 ml. of the standard sucrose + asparagine liquid medium was sterilized in 500 ml. Erlenmeyer flasks at 106° for 15 min. A modified medium with 10% mannitol (w/v) instead of the sucrose was also used.

Seed cultures. A spore suspension (2 to 6 × 10⁹ spores/ml.) was prepared from 14-day agar cultures and 1 ml. inoculated into each 500 ml. flask. Usually the fermentation was completed without further transfer, but in some experiments the first (seed) stage flasks were subsequently used as a source of inoculum for a second stage. Where mycelial inocula were used an aqueous suspension was prepared by crushing the sparsely sporing mycelium from 3-day agar cultures.

Flasks were incubated at 27° on a rotary shaker (Buck *et al.* 1968). For sampling, duplicate flasks were removed at intervals from the shaker and the course of fermentation was followed by the following parameters: microscopic examination and measurement of pH value, sugar utilization, nitrogen utilization, dry weight of mycelium and polysaccharide. All experiments were at least duplicated.

Analytical techniques

Mycelial dry weight. A sample of culture was centrifuged at 2500 g for 20 min. and the supernatant fluid removed. The mycelium was washed twice with distilled water, filtered onto a weighed filter paper and dried to constant weight at 70°. Highly viscous cultures were diluted 10 times before processing.

Polysaccharide. The supernatant fluid and washings from mycelial dry-weight determination were stirred with an equal volume of absolute ethanol and the precipitated polysaccharide collected on a glass rod and dried at 70° to constant weight.

Nitrogen. Samples obtained after distillation in a micro-Kjeldahl apparatus were assayed in a Technicon autoanalyser at 625 nm. using the alkaline phenol reagent of Garza & Weissler (1967).

Glucose was specifically determined by a glucose oxidase method modified for the autoanalyser by Marks & Lloyd (1963).

Sucrose was estimated as glucose by the above method after it had been hydrolysed at 37° for 30 min. by invertase (British Drug Houses, Poole, Dorset; 0.5 units/ml. of incubate).

Other analytical techniques used were substantially as described by Buck *et al.* (1968). The following changes and additions were made.

Protein estimation. Protein in solution was estimated by determining the extinction of the sample at 260 and 280 nm. The concentration was then obtained from a nomograph compiled by Adams (California Corporation for Biochemical Research) from the data of Warburg & Christian (1942).

Column chromatographic procedure. The method used was similar to that described by Chesters & Bull (1963). An aqueous slurry of 10 g. diethylaminoethyl (DEAE) cellulose was poured into a glass column and eluted with 200 ml. of N-NaOH to

remove impurities. The column was then washed until free from NaOH and equilibrated at 4° with 6 l. of 1 mM-phosphate buffer (pH 5.6); the final column size was 1.4 cm. diam. × 28 cm. The acetone powder containing the enzymes (60 mg.) was solubilized at 4° for 2 hr in 5 ml. of 1 mM-phosphate buffer (pH 5.6) and applied to the column. The column was eluted with a linear gradient of buffer at pH 5.6 increasing in ionic strength from 1 to 150 mM, prepared according to the method of Bock & Ling (1954); the flow rate was approx. 30 ml./hr; 5 ml. fractions were collected. The fractions were dialysed overnight at 4° against 1 mM-phosphate buffer (pH 5.6) and, after their protein content had been determined, were lyophilized.

Enzymic methods. Acetone powders of the enzymes in the culture medium were prepared at 4° as follows. After the mycelium had been removed, the culture medium was dialysed for 24 hr against 5 mM-acetate buffer (pH 5.2, 10 l./100 ml. of medium was used). The dialysed medium was then mixed slowly with two volumes of ice-cold acetone; glucan, when present, was removed at this stage with a glass rod. The protein from the resulting mixture was allowed to sediment overnight and was then isolated by decantation and centrifugation at 2500 g, and dried in vacuum over CaCl₂.

All enzyme reactions were made according to the recommendations of the International Union of Biochemistry. Reactions were done at 30° in 0.03 M-acetate buffer (pH 5.2). Substrate concentrations were 1% w/v, unless otherwise stated.

Glucanase activity was assayed by its action on the [U-¹⁴C]glucan and determining the radioactivity of the products (Buck *et al.* 1968).

The activity of β -glucosidase was determined by its action on *p*-nitrophenyl- β -D-glucopyranoside and measuring the liberation of *p*-nitrophenol at 410 nm. Also, β -glucosidase activity was assayed by its action on [U-¹⁴C]gentiobiose (prepared by the action of the glucanase on [U-¹⁴C]glucan) and on unlabelled gentiobiose, whence the product was determined by radioactivity and by glucose oxidase, respectively.

Sucrase activity *in vivo* was assayed by harvesting the fungal tissue, washing it briefly in 0.03 M-acetate buffer containing 5, 10 or 30% sucrose and then suspending it in its original culture volume in the same sucrose + buffer solution as used for the washing. The flasks were replaced on the shaker and 10 ml. samples taken over a period of 24 hr, filtered, and the sugars in the filtrate separated and identified by chromatography (Buck *et al.* 1968).

Preparative and counting techniques with ¹⁴C. In addition to counting from paper, liquid samples were also counted. These samples, obtained by fractionation procedures made on tissue (see below), were placed in liquid scintillant (6 g. of CIBA butyl-PBD scintillator + 50 g. naphthalene/l. toluene) and converted to a single-phase system by adding 2-methoxyethanol (methyl cellosolve).

Procedure for extraction of fungal tissue. The procedure followed was a modification of that described by Roberts *et al.* (1955) for *Escherichia coli*, using a ratio of 5 ml. extractant to 1 g. fresh weight of fungal tissue. The tissue was washed twice briefly in 0.01 M-acetate buffer, (pH 5.2); the washings were discarded and the washed tissue crushed in an X-press (AB Biox, Sweden). The crushed material was then extracted successively as follows: (a) For 10 min. at 4° in 5% trichloroacetic acid (TCA) to remove low molecular weight substances. (b) For 30 min. at 45° in 75% ethanol in water to remove lipid (and some protein). (c) For 15 min. at 45° in 75% ethanol (v/v) in water + ether (1 + 1) to remove remaining lipid. (d) For 30 min. at 95° in 5%

TCA to remove nucleic acid. (e) The residue, containing insoluble protein and polysaccharide, was washed in ether to remove TCA and dried in vacuum over CaCl_2 . The TCA was removed from the extracts by shaking them twice with ether.

Hydrolysis of solid residue. This was done at 110° overnight in 6 N-HCl in sealed tubes. Approx. 1 ml. HCl was used/5 mg. sample. The hydrolysates were neutralized with NaOH.

Fig. 1. *Claviceps fusiformis* strain 139/2/1G. Course of fermentation in shake-flasks: ●, mycelium; ■, polysaccharide; ▲, β -glucanase.

Fig. 2. *Claviceps fusiformis* strain 139/2/1G. Course of fermentation in shake-flasks (continued from Fig. 1): ○, sucrose; □, glucose; △, total nitrogen.

RESULTS

Course of fermentation of Claviceps fusiformis

The course of a typical fermentation of the *Claviceps fusiformis* strain used, during which glucan was autolysed, is shown in Fig. 1 and 2. The well-buffered medium remained in the range pH 5.2 to 5.8. Preliminary studies indicated an optimum spore inoculum of 2 to 8×10^8 spores/100 ml. medium; this gave well replicated growth and, subsequently, the most rapid glucan autolysis. Mycelial growth, as determined by dry weight, progressed through the usual lag and exponential phases, but within 1 to

2 days of entering the stationary phase a significant resurgence of growth occurred which coincided not only with decreasing viscosity related to glucan autolysis but also with release into the medium of nitrogenous products from the normal autolysis of the oldest cells. It is suggested that the nitrogen source, being no longer limiting, allowed a further short phase of growth for which ample glucose was available (see below).

During the first 3 to 4 days of growth germinating spores formed long thin hyphae which sporulated to give both micro- and macro-spores. Fragmentation of these sphacelial hyphae (Fig. 3), rapid growth, and the synthesis of glucan resulted in a thick

Fig. 3

Fig. 3. *Claviceps fusiformis* strain 139/2/1 G. Typical mycelial fragment from 4-day submerged culture showing few fat droplets in younger sphacelial cells and large fat reserves in older cells which have differentiated to a sclerotial form.

Fig. 4

Fig. 4. *Claviceps fusiformis* strain 139/2/1 G. Typical mycelial fragment from 7-day-old submerged culture composed solely of sclerotial cells.

viscous culture within 4 days of inoculation, after which sphacelial growth and sporulation declined. About 3 days after inoculation the older cells at the centre of the small groups of sphacelial hyphae became thicker, filled with fat droplets and progressed morphologically to resemble the compact plectenchymatic tissue of natural sclerotia, suggesting that this represented a diffuse change in growth form of this plant parasite from sphacelial to sclerotial similar to that occurring *in vivo*. Further, hyphal fragments also developed directly to form abundant short-branched sclerotial hyphae (Fig. 4). The initial autolysis of extracellular glucan was closely associated with the appearance of sclerotial cells in the culture, suggesting that the production of the factor responsible for glucan autolysis may be associated with sclerotial cells, whereas the glucan synthesis was clearly associated with sphacelial cells.

The sucrose content of the culture filtrate decreased rapidly, greatly in excess of that which one might expect to be required for growth and respiration alone. This discrepancy was investigated and the rapid disappearance of sucrose was shown to be due to the activity of a cell-associated sucrose. This enzyme split sucrose, liberating glucose only and forming oligofructosides by transfer. The liberation of free glucose was associated with the increase in mycelial dry weight and glucan. While the sucrose was being depleted, the glucose also diminished after the third day but did not disappear completely, because growth was then being limited by the very low asparagine content. Concurrently, glucose was also released by glucan hydrolysis which later provided the carbon source for the secondary growth phase from day 7, noted above.

Incorporation of glucose from the medium during the secondary growth phase was further demonstrated by injecting into flasks 150 μ C of [U- ^{14} C]glucose (3 ml.; 2.9 mC/mm), sterilized by Millipore filtration, on the 7th day and harvesting the flasks at

Fig. 5

Fig. 6

Fig. 5. Incorporation of ^{14} C from [U- ^{14} C]glucose into the mycelium of *Claviceps fusiformis* strain 139/2/1 G during late fermentation (autolysis of glucan): ■, culture filtrate; ●, mycelium and glucan; ○, respired (by subtraction).

Fig. 6. *Claviceps fusiformis* strain 139/2/1 G. Effect of age of seed stage inoculum on glucan content of second-stage fermentation: ■—■, 3-day-old inoculum; ■---■, 5 day-old inoculum; ■·····■, 7-day-old inoculum.

Table 1. *Claviceps fusiformis*, strain 139/2/1 G: incorporation of ^{14} C (μ C) from [U- ^{14} C]-glucose into various mycelial fractions during late fermentation (glucan autolysis).

[U- ^{14} C]glucose (3 ml. containing 150 μ C) was aseptically introduced into each flask during culture on the seventh day. Flask contents were harvested and fractionated as described in Methods. Respired values were obtained by subtraction.

Fraction	Time after introduction of [U- ^{14} C]glucose (days)					
	0	1	2	3	5	7
a	0	4.9	6.0	4.3	5.7	1.7
b	0	1.7	2.4	2.6	2.1	1.6
c	0	0.67	1.0	1.2	1.1	1.0
d	0	0.72	1.4	1.7	1.7	0.80
e	0	1.4	1.8	2.3	2.0	1.8
Spores	0	0.71	0.71	0.70	0.23	0.10
Glucan	0	1.1	0.70	0.43	0.21	0.34
Filtrate	150	71	46	40	24	33
Respired	0	68	90	97	113	110

intervals up to 14 days. The tissue was extracted as described above and assayed together with the culture medium for ^{14} C content. The results are given in Table 1, and Fig. 5, which show that the isotope was rapidly incorporated into all parts of the tissue to an extent that exceeded 10% by the 9th day.

Effect of age of seed stage on the glucan content during the second stage of fermentation

Seed flasks, inoculated as usual with spores, were grown for 3, 5 and 7 days and 10 ml. samples were transferred to duplicate second-stage flasks. Flasks inoculated with a 3-day seed showed an increase and then a decrease of glucan during fermentation (Fig. 6) similar to that occurring in the first stage (Fig. 1), whereas older inocula yielded progressively less glucan.

Comparison of spores and mycelia of different strains as inocula for optimum glucan autolysis

An optimum spore concentration ($> 10^9$ spores/ml.) for the inoculum of seed or first-stage flasks had been determined in preliminary investigations and this ensured reproducibility, good growth and a decline in glucan content after 5 days. An optimum (2×10^9 spores/ml.) and a low (2×10^4 spores/ml.) spore inoculum level were compared with non-sporing mycelial inocula comprising similar numbers of constituent cells/ml. For comparison the high glucan-yielding parent strain (139/2/1) was used at the same high inoculum levels (Table 2). A low spore inoculum had a longer lag phase and

Table 2. *Claviceps fusiformis* strain 139/2/1 G: growth and glucan content of 13-day shake cultures inoculated with spores or mycelial fragments at various concentrations

Inocula prepared as described in Methods					
Strain	Inoculum	Mycelium (mg./ml. culture)	Polysaccharide (mg./ml. culture)	pH value	Morphology
139/2/1 G	2×10^9 spores	14.9	0.90	6.2	sclerotial
	2×10^4 spores	11.2	1.44	5.5	sclerotial where growth good: sphaelial where growth poor
	2×10^9 mycelial	13.4	1.14	5.8	sclerotial
	2×10^4 mycelial	16.3	2.08	5.9	sclerotial
	2×10^9 spores	24.0	7.7	5.4	mainly sphaelial

yielded results not reproducible, whereas a similar number of cells in mycelial form had a much shorter lag phase and subsequently grew well. The viscosity of fermentations of strain 139/2/1 G after 5 to 7 days incubation indicated vigorous glucan formation and, whether established from spore or mycelial inocula, the glucan content after 13 days represented the residue remaining after considerable autolysis of the polysaccharide. There was a correlation between the existence of a sclerotial growth-form during the latter stages of the fermentation and the autolysis of glucan. Conversely, strain 139/2/1, in which no noticeable glucan autolysis occurred, grew principally in a non-sclerotial or sphaelial form in spite of an inoculum which, in the autolysing strain, was optimum for glucan autolysis. The ability to autolyse glucan during the latter stage of fermentation was principally a characteristic of the new strain but was best expressed where a high inoculum was used.

Enzyme investigations

Action of extracellular enzymes on carbohydrates. The extracellular enzymes extracted as an acetone powder were incubated at a final concentration of 0.25% with several carbohydrates individually, and samples were taken over periods up to 24 hr. The extracellular enzymes hydrolysed maltose, β -glucosides and β 1 $\rightarrow$ 3 linked glucans; 10% of the maltose was hydrolysed per hour, the β -glucosides were almost completely hydrolysed in 2 h; the glucanase activity was 5 to 10% of that of the exo- β 1 $\rightarrow$ 3 glucanase (Reese & Mandels, 1959; see Buck *et al.* 1968) when incubated under the same conditions. There was also a noticeable action of the enzyme mixture on sucrose; by 24 hr approx. 50% of the original substrate had been broken to glucose and oligosaccharides. Since no fructose was detected, the action appeared to be that of a sucrase giving fructosylsucroses by transfer (see above) rather than an invertase which produced mainly glucose and fructose. The pH optimum was broad in each case: for the maltase, β -glucosidase, and β -glucanase it was between pH 5.0 and 5.5 and for the sucrase between pH 5.5 and 6.0.

Table 3. *Claviceps fusiformis* 139/2/1 G: comparison of the effect of sucrose and mannitol as carbon sources on the yields of mycelium and polysaccharide and on the activity of extracellular enzymes

Cultures were harvested at 8 days and acetone powders of the extracellular enzymes prepared. The enzymes were assayed at 30° with 1% (w/v) substrate and 0.1% (w/v) acetone powder. Samples were taken at intervals over 5 hr and assayed as in Methods. Results are expressed as % of substrate (cellobiose for β -glucosidase and glucan for β -glucanase) hydrolysed/hr/mg. acetone powder.

Carbon source	Mycelium (mg./ml. culture)	Polysaccharide (mg./ml. culture)	Extracellular material (mg. acetone powder)	β -glucosidase activity	β -glucanase activity
Sucrose	10.9	2.52	5.0	38	3.3
Mannitol	8.06	0.39	12.5	35	3.5

Activity of extracellular enzymes from cultures producing no glucan. The acetone powders used above were all prepared from 'sucrose' cultures which produced glucan. To determine whether or not the enzymes were induced or constitutive the organism was grown on the mannitol medium in which little or no glucan was produced (Table 3). These were compared with glucan-producing cultures grown concurrently and the acetone powders were assayed as above for the relevant enzymes: β -glucosidase and β -glucanase. The results are given in Table 3. Low polysaccharide production did not correspond to low enzyme production. No significant differences in the specific activities of the two enzyme preparations were noted, although the yield of acetone powder from 100 ml. of each culture was more than double for the mannitol medium (in a typical case: 5 against 12.5 mg.). This suggests that part of the difference in unit weight of glucan produced in the two cultures may have been due to a greater rate of breakdown in the mannitol medium, and indicates that the enzymes are constitutive.

In preliminary investigations with two *Claviceps purpurea* strains in which no glucan was detected when cultured under conditions similar to *C. fusiformis*, amounts of β -glucanase activity even higher than those found in *C. fusiformis* were found in the culture medium after only 2 to 3 days growth.

Polysaccharide bound enzyme. During these experiments it was observed that when glucan isolated from the cultures was incubated alone with β -glucosides, the latter were slowly broken down ($\approx 10\%$ substrate hydrolysed/hr). This phenomenon was investigated further. The polysaccharide from above was heated in aqueous suspension for 5 min. in a water bath at 100° . Following this treatment all activity towards β -glucosides was lost, indicating the presence of polysaccharide-bound enzyme. To determine the intensity of the binding the polysaccharide was first subjected to a rigorous re-purification by the procedure described by Buck *et al.* (1968): six precipitations were done. The final product had substantially the same specific β -glucosidase activity as above.

Secondly, 50 mg. polysaccharide was suspended and gently stirred for 6 hr at room temperature in 5 ml. 0.03 M-acetate buffer (pH 5.2). The polysaccharide was then precipitated from the resulting viscous solution with ice-cold acetone as in the purification procedure above. The precipitated polysaccharide was removed and lyophilized; the precipitate from the slightly cloudy supernatant fluid left was isolated by centrifugation at 500 g and dried in vacuum over CaCl_2 to yield about 1 mg. product. The resulting polysaccharide had substantially the same activity as initially; the precipitate from the supernatant fluid had only a trace of β -glucosidase activity ($< 0.5\%$ substrate hydrolysed/hr). A sample of the precipitate itself was then subjected to the action of the $\text{exo-}\beta 1 \rightarrow 3$ glucanase (see above); it was not digested and no glucose or gentiobiose was detected by thin-layer chromatography. This indicated that the precipitate was not composed of small fragments of polysaccharide and that its activity was probably due to traces of enzyme freed from the polysaccharide. Repeating this extraction procedure in the presence of 2% cellobiose, a substrate for the enzyme, did not increase the yield or activity of free enzyme.

Lastly, in an attempt to free the enzyme from polysaccharide and to determine whether it remained active when in free solution and had properties similar to the soluble β -glucosidase, a sample of the polysaccharide (20 mg.) was treated with the $\text{exo-}\beta 1 \rightarrow 3$ glucanase (2 mg.) in 2 ml. 0.03 M-acetate buffer, (pH 5.2), for 6 hr at 30° . The product was then dialysed at 4° overnight against 1.5 l. of the same buffer, centrifuged to remove any debris, and the soluble protein content determined. No volume change occurred during dialysis and the protein content, over and above that due to the $\text{exo-}\beta 1 \rightarrow 3$ glucanase, had risen by about 100 μg . Acetone precipitation was then done as before to remove any residual polysaccharide. Cellobiose was over 50% hydrolysed by the resulting acetone powder within 1 hr, which was slightly slower than the free enzyme (β -glucosidase activity of the $\text{exo-}\beta 1 \rightarrow 3$ glucanase alone was undetectable at 1 hr). With *p*-nitrophenol- β -glucoside as a substrate, the enzyme had a K_m of 6.6×10^{-4} M; in contrast the free enzyme had a K_m of 2.9×10^{-4} M (with the 'natural' substrate, namely gentiobiose, the K_m obtained was 3.1×10^{-3} M). In the latter case marked substrate inhibition occurred at 4 mM with the *p*-nitrophenol salt, and at 2.5 mM with gentiobiose. Bound β -glucosidase was considerably more stable than the unbound enzyme. This was shown by repeated (4 to 6) precipitations with ethanol at room temperature. Under these conditions the unbound material lost more than 50% of its activity, whilst the bound enzyme was virtually unaffected.

Development of extracellular enzymes during culture. Acetone powders of the extracellular enzymes were prepared at intervals during the course of a fermentation starting on the 3rd day and finishing on the 13th day. Other variables in the culture,

namely: pH value, glucose, sucrose, asparagine, total nitrogen and dry weight of mycelium, polysaccharide and acetone powder were assayed concurrently (see Fig. 1, 2). The specific glucanase activity of the acetone powder was determined by the use of [^{14}C]glucan prepared as by Buck *et al.* (1968). Hydrolysis of the [^{14}C]glucan at 2.5% (w/v) was done with 0.5% of acetone powder in each case. Initial rates of reaction were determined by assaying [^{14}C]glucose liberated during the hydrolysis at intervals up to 20 hr. As the only product was glucose in all cases, the results indicated that β -glucosidase was being formed concomitantly with the glucanase, the hydrolytic step of the latter being rate-limiting; a correction for β -glucosidase activity was not applied in this case (see below). The results are included in Fig. 1 and Table 4 and show that enzyme activity appeared early in the culture and consistently increased, specifically and totally.

Table 4. *Claviceps fusiformis* strain 139/2/1 G:
development of β -glucanase activity during culture

Cultures were harvested and acetone powders of the extracellular enzymes prepared. The β -glucanase was assayed at 30° using 2.5% (w/v) [^{14}C]glucan and 0.5% (w/v) acetone powder in each case.

Day	Extracellular material (mg./ml. of culture)	Specific activity of β -glucanase ($\mu\text{g. glucose produced/min./mg. extracellular material}$)	Total activity (units*/100 ml. culture)
3	0.04	2.7	10.8
4	0.06	5.6	33.6
6	0.16	5.7	91.2
9	0.48	6.6	316.8
10	0.79	7.0	553.0
11	0.90	8.0	720
13	0.80	11.0	880

*1 unit = 1 $\mu\text{g. glucose produced/min.}$

Characterization of the glucanase in the extracellular enzyme mixture. To characterize the glucanase further it was necessary to separate it from the β -glucosidase which was also present in the enzyme mixture. This was done by column chromatography. The specific activity of the protein in the column fractions obtained was determined by assaying with [^{14}C]glucan for the glucanase as described above. As with the $\text{exo-}\beta 1 \rightarrow 3$ glucanase (see above), two products were obtained, which were identified as glucose and gentiobiose by the methods described by Buck *et al.* (1968). Hence the enzyme activity was most conveniently expressed as $\mu\text{mole product liberated/hr./ml. incubate/mg. protein}$. The specific activity of the β -glucosidase in the column fractions was determined by its action on 1.5 mM-*p*-nitrophenyl- β -glucoside as described above. The results are shown in Fig. 7.

Assaying the enzymes at 30° increased the ratio of glucose to gentiobiose over that obtained at 50° with the $\text{exo-}\beta 1 \rightarrow 3$ glucanase for the same sample of glucan (from 2.0 to 2.1:1). The lowering of the temperature therefore appeared to give the β -glucosidase, known to be present in the $\text{exo-}\beta 1 \rightarrow 3$ glucanase, a selective advantage with respect to activity. As the lowest ratio of products, obtained at 50°, was maintained for over 5 hr, it may be concluded that β -glucosidase activity was suppressed and that this ratio is the correct value, particularly since it also agrees with the non-enzymic estimations (Buck *et al.* 1968). Therefore to obtain a true value for the glucan-

ase activity (total number of product molecules liberated) of all samples for *Claviceps fusiformis* it was necessary to correct for the β -glucosidase activity, assuming a true value of 2:1 for the products; this was done for Fig. 7. The lowest product ratios obtained were in fractions 7 and 8 (2:1 and 2:4:1, respectively) which were therefore substantially free from β -glucosidase activity. The activity of the β -glucosidase for fractions 9 and above, whilst specifically very low, was, in terms of total activity, significant. By fraction 16 the ratio had risen to over 7:1.

Fig. 7. Column chromatography, on DEAE cellulose, of extracellular enzymes produced by *Claviceps fusiformis* strain 139/2/1 G during fermentation: ●, protein; Δ, β -glucanase; □, β -glucosidase.

Table 5. *Claviceps fusiformis* strain 139/2/1 G: comparison of activities of the crude and purified enzyme from *C. fusiformis* with the $\text{exo-}\beta\text{I} \rightarrow 3$ glucanase

The enzymes (0.5%, w/v) were incubated at 30° with [^{14}C]glucan (2.5%, w/v) and the products separated as described in Methods. The product levels at 6 hr for the $\text{exo-}\beta\text{I} \rightarrow 3$ glucanase indicate 80% substrate utilization.

	Time of incubation (hr)			
	1	2	3	6
	$\mu\text{moles of product liberated/ml. incubate/mg. enzyme}$			
Crude enzyme				
Glucose	0.95	2.08	3.32	6.60
Gentiobiose	0.005	0.02	0.05	0.09
Purified enzyme (fraction 9)				
Glucose	2.60	4.85	6.95	11.50
Gentiobiose	0.85	1.55	2.20	3.31
Exo- $\beta\text{I} \rightarrow 3$ glucanase				
Glucose	8.14	10.93	12.02	13.28
Gentiobiose	3.72	4.83	5.12	5.56

Comparison of the purified glucanase with the $\text{exo-}\beta\text{I} \rightarrow 3$ glucanase. Since the purified glucanase (fraction 9 was used) gave the same products as the $\text{exo-}\beta\text{I} \rightarrow 3$ glucanase of

Reese & Mandels (1959) when acting upon the glucan from *Claviceps fusiformis*, a direct comparison of the two enzymes, together with the original unpurified acetone powder mixture was made. Each of the three was incubated in the usual way with [^{14}C]glucan. Samples were taken over a period of 6 hr; the results are given in Table 5. The difference in the ratios of glucose to gentiobiose between $\text{exo-}\beta\text{1}\rightarrow\text{3}$ glucanase and the purified glucanase from *C. fusiformis* may be ascribed to the greater β -glucosidase activity of the latter. From Table 5 it can be seen that, after applying the correction value as above, the column chromatography of the acetone powder mixture resulted in a purification of the glucanase by 4.5 times for fraction 9.

DISCUSSION

The subsequent autolysis of the extracellular glucan produced during the early stage of fermentation of the novel strain 139/2/1G of *Claviceps fusiformis* has been shown to be due to the activity of an $\text{exo-}\beta\text{1}\rightarrow\text{3}$ glucanase of the type described by Reese & Mandels (1959) and is the first reported occurrence of this phenomenon in *Claviceps* spp.; a similar phenomenon has also been detected in *C. purpurea*. The extent of the enzyme activity, which began 2 to 3 days after inoculation and was paralleled by the concentration of the enzyme in the culture filtrate, followed a sigmoid pattern reaching a maximum within about 12 days. Characterization of the glucanase has confirmed the speculations of Davis *et al.* (1965) that an 'exocellular glucanase' could account for the hydrolysis of extracellular glucans observed in fermentations of their fungi. Production of the glucanase was accompanied by the formation of several other enzymes, the most notable of which was a highly active β -glucosidase; this occurred both free and glucan-bound. The latter form rendered the enzyme more stable though decreasing its affinity for the substrate. However, the K_m of both forms was sufficiently low to make it impossible to detect in the culture medium any products of glucan hydrolysis other than glucose. The glucose, thus liberated from the glucan, mixed with that which was liberated from sucrose during the first 4 days of incubation by the activity of another enzyme, a cell-associated sucrase. Towards the end of the fermentation this free glucose was partly utilized for a second growth phase.

This mechanism for the release of glucose from β -glucans, which have also been shown to be present in the naturally occurring sclerotia of *Claviceps fusiformis* (Buck *et al.* 1968), may enable these polysaccharides to constitute a reserve carbon source for the sclerotial germination process in addition to the large reserves of triglyceride oil (Mantle, 1968). Not only is the glucanase of intrinsic interest as a mould metabolite but its activity in restoring low viscosity during *C. fusiformis* fermentations has already facilitated the production and easy extraction of large yields of clavine alkaloids in 300 l. fermenters in this department. The usual *C. fusiformis* strains which do not produce the glucanase (Buck *et al.* 1968) are limited early in the fermentation by aeration deficiency, and any alkaloid product can only be extracted after the glucan has been precipitated from the culture and filtered off with considerable difficulty.

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