The Involvement of Cellulase and Laminaranase in the Formation of *Pythium* Protoplasts

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SUMMARY

Five inducible streptomycete lytic enzyme complexes were equally capable of producing protoplasts from a *Pythium* sp. strain PRL 2142, when compared on a quantitative basis. Both cellulase and laminaranase from various microbial sources were required to produce the protoplasts under standard conditions. Protease was not required; lipase shortened the survival time of protoplasts. It was concluded from an analysis of the products of enzymic hydrolyses that *Pythium* sp. PRL 2142 cell wall contained cellulose, β -D-(1 $\rightarrow$ 3) glucan, and a β -D-(1 $\rightarrow$ 3) glucan possessing D-glucose units attached by β -(1 $\rightarrow$ 6)-linkages.

INTRODUCTION

Glycosidases, lipases and proteases have all been shown to be involved in the formation of fungal 'protoplasts'. However, little quantitative data is available in published form about these enzymes, especially with regard to the degradation of cell wall of oomycetous genera (Villanueva, 1966). The cell walls of these genera are known to possess both cellulose and β -D-(1 $\rightarrow$ 3) glucans and associated gentibiose components (Aronson, Cooper & Fuller, 1967; Bartnicki-Garcia & Lippmann, 1967; Wang & Bartnicki-Garcia, 1966). Streptomycete enzyme systems capable of producing 'protoplasts' from *Pythium* sp. PRL 2142 have been described by Sietsma, Eveleigh, Haskins & Spencer, (1967). A qualitative and quantitative analysis of these lytic enzyme systems is now presented; attention is focused on the cellulase, laminaranase, lipase and protease components. Several components of the cell wall are now identified by enzymic techniques.

METHODS

The preparation of the streptomycete lytic enzyme complexes used in the production of *Pythium* 'protoplasts' has been described by Sietsma *et al.* (1967). Other enzymes, used comparatively, included a *Streptomyces* sp. B 814 cellulase (E.C. 3.2.4.4, from E. T. Reese: Reese & Mandels, 1963), *Aspergillus niger* cellulase, subtilisin (E.C. 3.4.4.16; from Nutritional Biochemical Corporation, Cleveland, Ohio), *Rhizopus arrhizus* laminaranase (E.C. 3.2.1.6; Reese & Mandel, 1963), bacterial laminaranase (given by Glaxo Laboratories, England: see Manners & Patterson, 1966), trypsin (E.C. 3.4.4.4; from Armour Laboratories, Chicago, Illinois), wheat germ lipase (E.C. 3.1.1.3; from Pentex, Kankakee, Illinois), purified pork pancreatic lipase (given by C. G. Youngs, P.R.L., N.R.C., Saskatoon).

Substrates used. These were as follows: laminaran (β -D-(1 $\rightarrow$ 3)-glucan) and nigeran (mixed α -D-(1 $\rightarrow$ 3), α -D-(1 $\rightarrow$ 4)-glucan) (Koch-Light Labs., Colnbrook, England); carboxymethylcellulose (CMC-cellulose—courtesy Hercules Trading Corp., Wilmington, Delaware, U.S.A.); pustulan (β -D-(1 $\rightarrow$ 6)-glucan; from *Umbilicaria pustulata*—given by E. T. Reese). Purified chitan (β -(1 $\rightarrow$ 4) linked 2 acetamido-2-deoxy-D-glucan-crustacean shell) and crown gall (β -(1 $\rightarrow$ 2)-D-glucan from *Agrobacterium tumefaciens* IIBV7) were available at this laboratory. Pythium cell-wall preparations were made by boiling the mycelium (7-day culture) in water for 10 min. to inactivate autolytic enzymes, and then homogenizing it in a Waring blender followed by ultrasonic treatment. The wall preparations were washed three times with water. Although only 50% breakage of the mycelium was achieved, its coenocytic nature allowed complete loss of cytoplasm as judged by random microscopic examination.

The glycosidases of the lytic enzyme preparations were investigated by reacting enzyme solution (0.1) with a range of polysaccharides (3 mg./ml.; 0.1 ml.) for 16 hr at room temperature. Thiomersalate 0.005% was used as a bacterial inhibitor. The reactions were terminated by heating at 100° for 10 min. The degree of hydrolysis and the lytic products were characterized by paper chromatography (solvent c—see below). The enzyme preparations were quantitatively tested for β -D-(1 $\rightarrow$ 3)-glucanase (laminaranase) and cellulase (Cx) by using laminaran and carboxymethylcellulose as substrates respectively. The assays were done with 0.5 ml. substrate (5 mg./ml.) + enzyme and 0.05 M-sodium acetate buffer (pH 5.8) to 1.0 ml. at 30°, for 15 (or occasionally 30) min. The reactions were allowed to run to a maximum of 5% substrate degradation in order to maintain a stoichiometric response between the product and the amount of enzyme added (Eveleigh, 1967; Miller, Blum, Glennon & Burton, 1960). An enzyme unit is defined as that amount of enzyme which released 1 μ mole glucose equivalent/min. at 30°. Reducing end-groups (as glucose equivalents) were estimated by the Nelson-Somogyi method (Neish, 1952). Initially cellulase assays were made on CMC-cellulose 0.7 degree of substitution (DS); later assays were made on the 0.4 DS CMC-cellulose which is four times more reactive than the 0.7 DS substrate. The data for the cellulase assay were presented on the basis of this latter substrate. Lipase was determined titrimetrically (Marchis-Mouren, Sarda & Desnuelle, 1959). Olive oil was used as a standard and one lipase unit defined as that amount of enzyme which released 10 μ -equiv. acid/min. under standard conditions (Marchis-Mouren *et al.* 1959). Proteolytic activity was determined by using the protein-dye complex azocoll, as substrate (*Calbiochem. tech. Bull.* 1966); a unit is defined as 0.001 extinction change/min. at 37°, pH 7.5. Proteins were determined by the Folin phenol method (Lowry, Rosenbrough, Farr & Randall, 1951) with bovine serum albumin as a standard.

Paper chromatographic analysis was done on Whatman filter paper no. 1 with the following solvent systems: (a) *n*-butanol + ethanol + water (3 + 1 + 1 by vol.); (b) ethyl acetate + pyridine + water (10 + 4 + 3 by vol.); (c) ethyl acetate + acetic acid (glacial) + water (9 + 2 + 2 by vol.); (d) ethyl acetate + formic acid + acetic acid + water (18 + 1 + 3 + 4 by vol.). Reducing compounds were detected with *p*-anisidine. Partial acid hydrolysis of the cell walls was done in 0.4 N-H₂SO₄ at 100° for periods of 20 min., 1 hr and 3 hr. A more complete hydrolysis was performed in N-H₂SO₄ at 100° for 20 hr. Hydrolysates were neutralized with BaCO₃, filtered and concentrated *in vacuo* at room temperature.

RESULTS

The initial survey of enzymes present in the crude lytic streptomycete system was done by chromatographic analysis of enzymic hydrolysates and results are presented in Table 1. Endo- β -D-(1 $\rightarrow$ 3)-glucanase was shown to be present in each system by the production of β -(1 $\rightarrow$ 3)-D-linked-oligosaccharide series as degradation products from laminaran. Exo-laminaranase was also shown to be present in three systems (Table 1) by their degradation of a *Claviceps* sp. PRL 1980 glucan (Perlin & Taber, 1963) to yield glucose and gentiobiose. The presence of both endo- and exo-laminaranases in the *Streptomyces* sp. 0143 complex was also demonstrated by an n.m.r. analysis of the glucose and oligosaccharide anomers released initially during enzymic degradation of laminaran (i.e. α -glucose and β -laminaribiose + β -laminaritriose—unpublished observations). Cellulase and β -glucosidase were present in each system, though lower levels of the latter enzyme were present in the *S. albidoflavus* system. The activity of two systems towards pustulan is noteworthy. The α -glucosidases present in these systems are probably constitutive, as starch has not been reported from *Pythium* mycelium, and only glucose and mycelium were used for the induction of the lytic enzymes.

Table 1. *Glycosidase activity of the streptomycete lytic complexes*

Substrate	<i>Strepto- myces</i> sp. 0143	<i>S. satsu- maensis</i> 1399	<i>Strepto- myces</i> sp. 1294	<i>S. globi- sporus</i> B 2872	<i>S. albedo- flavus</i> A 14
Cellobiose	+	++	++	++	+
Laminaran	++	++	++	++	++
Cellulose (CMC)	++	++	++	+	+
Lichenan	++	++	++	++	+
<i>Claviceps</i> sp. PRL 1980 glucan	+	++	+	nt	nt
Pustulan	o	+	o	+	o
Crown gall	o	o	+	o	o
Chitan	o	o	o	o	o
Maltose	++	+	o	+	o
Starch	++	++	+	o	o
Nigeran	o	o	o	o	o

++ , Strong positive reaction; + , positive reaction; o , negative reaction; nt , not tested.

This initial survey was followed by a more detailed analysis of the laminaranase and cellulase activities of the preparations. Lipase and protease were also determined because Acha, Aguirre, Lopez-Belmonte, Villanueva (1966) and Tabata & Terui (1963), respectively, reported their involvement in 'protoplast' production. The results are presented in Table 2.

It was previously reported (Sietsma *et al.* 1967) that *Streptomyces* sp. 0143 and *S. globisporus* lytic enzymes were capable of producing 'protoplasts' from *Pythium* species, while those from *S. satsumaensis* caused extensive lysis only. A strong correlation is seen between those results and the results presented in Table 2, i.e. systems producing the greatest lysis possessed generally greater amounts of laminaranase and cellulase. The *S. satsumaensis* system also had a major lipase component. The failure to form protoplasts by this system is probably due to the action of this lipase component, as the addition of wheat germ lipase to the other positive systems causes lysis of proto-

Table 2. Major enzymes present in the streptomycete lytic complexes

Protein (mg./ ml.)	$\beta(1,3)$ -Glucanase		$\beta(1,4)$ -Glucanase		Lipase		Protease	
	Units/ ml.	Specific activity	Units/ ml.	Specific activity	Units/ ml.	Specific activity	Units/ ml.	Specific activity
<i>Streptomyces</i> sp. 0143	1.68	1.245	0.740	0.492	0.0155	0.0092	12.1	7.2
<i>S. satsumaensis</i> 1399	2.42	0.363	0.150	0.532	0.0288	0.0119	115	47.5
<i>Streptomyces</i> sp. 1294	3.88	0.486	0.125	0.620	0.0119	0.0031*	0	0
<i>S. globisporus</i> B-2872	2.53	0.335	0.132	+	—	—	—	—
<i>S. albidoflavus</i> A-14	4.12	0.963	0.234	0.052	0.0875	0.0212	206	50.0
Cellulase (Cx) (<i>Streptomyces</i> sp. B-814)	0.11	0.00026†	0.0024	0.14	0.0242	0.2200	0	0
Cellulase (Cx) (<i>Aspergillus</i> <i>niger</i>)	0.56	0.03	0.053	0.46	0.0256	0.0457	0	p
Laminaranase (bacterial)	0.41	0.043	0.105	0.036	0.0342	0.0845	15.9	38.8
Laminaranase (<i>Rhizopus</i> <i>arrhizus</i>)	0.65	0.040	0.62	0.002	0.0057	0.0867	0	0
Lipase (pork pancreatic)	1.30	—	—	—	0.4000	0.3077	—	—
Lipase (wheat germ), pH 9.0	0.84	—	—	—	0.0266	0.0269	—	—
Lipase (wheat germ), pH 7.4	0.84	—	—	—	0.0051	0.0061	—	—
Lipase (wheat germ), pH 6.2	0.84	—	—	—	0	0	—	—
Trypsin	0.447	—	—	—	—	—	151	338
Subtilisin	1.366	—	—	—	—	—	1620	1186

*3 hr assay. †2.5 hr assay. —, not tested; +, not tested quantitatively.

plasts (Sietsma *et al.* 1967). Two of the systems (*S. satsumaensis*, *S. albidoflavus*) possessed marked protease activity. These proteases were assumed to possess limited specificity from a comparison of their reaction kinetics with those of trypsin and subtilisin and other proteases (*Calbiochem. tech. Bull.* 1966).

The streptomycete lytic enzymes were compared in their ability to produce protoplasts on a quantitative basis with regard to laminaranase activity. The induction of protoplast formation was done as previously described (Sietsma *et al.* 1967) using 50 mg. mycelium (equiv. 5 mg. dry wt). The lytic enzymes were added at an equivalent of 0.621 unit laminaranase, i.e. a concentration at which the *Streptomyces* sp. 0143 preparation (0.5 ml.) showed good protoplast formation. Three streptomycete preparations produced protoplasts after 24 hr incubation at 30°, while that from *S. satsumaensis* caused extensive lysis only (Table 3). The *S. albidoflavus* preparation also produced protoplasts, but after continued incubation (36 hr). The slower action of this

Table 3. *Comparative amounts of laminaranase and cellulase used for protoplast formation*

	Laminaranase (unit)	Cellulase (unit)	Protoplast produced after
<i>Streptomyces</i> sp. 0143	0.621	0.246	24 hr
<i>S. satsumaensis</i>	0.621	0.910	Complete lysis in 24 hr
<i>Streptomyces</i> sp. 1294	0.621	0.784	24 hr
<i>S. globisporus</i>	0.621	NT	24 hr
<i>S. albidoflavus</i>	0.621	0.032	36 hr

latter preparation was thought to be due to a suboptimal concentration of Cx cellulase. Further trials with various amounts of enzyme (maximum 0.096 Cx units), which included successive dosing with the lytic preparation every 12 hr, did not shorten the incubation period of 36 hr. Thus the original preparation apparently did not lack sufficient Cl cellulase and 0.1 unit Cx cellulase is probably a suboptimal amount of enzyme.

The preparation of *Pythium* protoplasts was further investigated by using microbial laminaranases and cellulases from various sources. The activities of these preparations are reported in Table 2. Equivalent amounts of the two laminaranases (0.75 unit) and of the two cellulases (0.5 unit) were added to the standard protoplast-producing incubation system, both singly and together. Samples were examined periodically by phase-contrast microscopy. The results are presented in Table 4. A good correlation is seen between the production of protoplasts and the combined use of laminaranase and cellulase. One exception was found in that the *Streptomyces* sp. B 814 cellulase, which had a low value of laminaranase, did produce a few protoplasts after 48 hr incubation. The deleterious effect of high concentrations of lipase on protoplast stability was again noticed when bacterial laminaranase or wheat germ lipase was used.

Hydrolysis of *Pythium* cell walls with dilute sulphuric acid produced glucose (major), laminaribiose and cellobiose, these sugars being identified chromatographically in three solvent systems (a, b, c). The cell wall was further characterized by enzymic degradation by using the streptomycete lytic preparations. The major products released were glucose, laminaribiose and gentiobiose; cellobiose, laminaritriose,

laminaritetraose and cellotriose were minor products, with a trace of 4- α - β -D-laminaribiosyl-D-glucose. Laminaritriose, laminaritetraose, cellobiose 4- α - β -D-laminaribiosyl-D-glucose and 3- α - β -D-celliosyl-D-glucose can be separated chromatographically (solvent, d, quadruple development). Neither of the latter two compounds were produced in appreciable amounts during time-sequence enzymic cell-wall analysis, whereas the other oligosaccharides did gradually accumulate in spite of the presence of a β -glucosidase component in the lytic systems. This would indicate that the *Pythium* sp. PRL 2142 cell walls do not possess a major mixed-linkage β -D-glucan component of the lichenan type. The cell walls on hydrolysis with a purified basidiomycete exo- β -(1 $\rightarrow$ 3)-D-glucanase (Reese & Mandels, 1963) yielded both glucose and gentiobiose. Thus a glucan consisting of a β -D-(1 $\rightarrow$ 3)-linked main chain with D-glucose units attached as branches by β -D-(1 $\rightarrow$ 6)-linkages, similar to that obtained from a *Claviceps* sp., PRL 1980 (Perlin & Taber, 1963) appeared to be present.

Table 4. *Production of Pythium protoplasts by synergistic action of laminaranase and cellulase*

Expt.	Enzymes	Amounts of Enzymes (units)		Protoplast forma- tion
		Laminaranase	Cellulase	
1	<i>Rhizopus arrhizus</i> laminaranase	0.75	0.038	—
2	Bacterial laminaranase	0.75	0.628	+ + *
3	<i>Aspergillus niger</i> cellulase	0.033	0.5	—
4	<i>Streptomyces</i> sp. B 814 cellulase	0.0009	0.5	+
5	<i>R. arrhizus</i> laminaranase + <i>A. niger</i> cellulase	0.783	0.538	+ +
6	<i>R. arrhizus</i> laminaranase + <i>Streptomyces</i> sp. B 814 cellulase	0.7501	0.538	+ + +
7	<i>R. arrhizus</i> laminaranase + <i>Streptomyces</i> sp. B 814 cellulase + wheat germ germ lipase (0.5 mg)	0.7501	0.538	+ + + *
8	Bacterial laminaranase + <i>Streptomyces</i> sp. B 814 cellulase	0.7501	1.128	+ + *

+, Few protoplasts (48 hr); ++, numerous protoplasts (44 hr); + + +, numerous protoplasts (22 hr).

* Protoplasts lysed relatively rapidly.

Endo- β -D-glucanases do not attack this type of glucan. Hence the presence of laminaribiose (major) and cellobiose as products from the action of the streptomycete lytic complexes on *Pythium* cell walls indicated the presence of an unsubstituted β -D-(1 $\rightarrow$ 3) glucan as the major, and cellulose as a minor, component of these walls. The large amounts of gentiobiose produced in such hydrolyses may have arisen from the β -D-(1 $\rightarrow$ 6) substituted β -D-(1 $\rightarrow$ 3) glucan, for the lytic complexes from the *Streptomyces* sp. strains 0143, 1294 and 1399 were shown to possess an exo-glucanase by their positive action on the β -D-(1 $\rightarrow$ 6) substituted *Claviceps* glucan (Perlin & Taber, 1963). There still remains the possibility that the gentiobiose arose from a β -D-(1 $\rightarrow$ 6) glucan. Pustulanase activity was observed in two of the lytic preparations (Table 1). An alkali insoluble glucan fraction has been reported from *Phytophthora cinnamomi* which contained either separate β -D-(1 $\rightarrow$ 3) and β -D-(1 $\rightarrow$ 6)_n homopolymers or a mixed linkage polymer (Bartnicki-Garia & Lippman, 1967).

DISCUSSION

Streptomycete lytic complexes that are capable of lysing the cell walls of the *Pythium* sp. PRL 2142 have been shown to possess, as major components, exo- and endo-laminaranases and a cellulase. Initially we reported that only two of the five lytic preparations were capable of producing 'protoplasts' (Sietsma *et al.* 1967). However, when these preparations were compared quantitatively with respect to laminaranase activity four of the preparations produced 'protoplasts', while the other caused complete lysis (Table 3). The amounts of enzyme cited allowed the ready formation of protoplasts under our conditions in 24 hr. Much smaller amounts of these enzymes plus cellulase will produce some protoplasts. The lowest amount of cellulase (Cx) necessary for protoplast formation appeared to be 0.1 unit from the experiments carried out with the *Streptomyces albidoflavus* complex. The amount of laminaranase generally used 0.621–0.75 unit (Tables 3, 4) was probably excessive, since the *Streptomyces* sp. B 814 cellulase (0.5 unit) which contained only 0.00093 unit of laminaranase was still capable of forming a few protoplasts in 24 hr (Table 4). Laminaranase 0.00093 unit can release 240 μ g. glucose-equivalent in 24 hr and is quite sufficient, theoretically, to produce protoplasts in this system, especially if the enzyme action was localized. The *Pythium* sp. PRL 2142 possesses a laminaranase (unpublished observation), and the possibility also exists that this enzyme may be activated by the addition of the streptomycete cellulase preparation. An anomaly is seen, however, in that *Aspergillus niger* cellulase (0.5 unit) containing 0.038 unit laminaranase was not capable of producing *Pythium* protoplasts. Other enzymes may be essential; for instance the presence or even necessity of a Cl cellulase (Mandels & Reese, 1964) has not been established. Several other factors will also have to be determined (e.g. product inhibition) before the system can be put on a rigorous quantitative basis. At present, a comparison of the amounts of the enzymes used with those of other workers is not informative because of variations in assay procedures and organism studied. A general inference is that in this study smaller amounts of enzyme and longer incubation periods have been used than by other workers (Lloyd, Noveroske & Lockwood, 1965; Mitchell & Alexander, 1963). Bartnicki-Garcia & Lippman (1967) used the same *Streptomyces* sp. B 814 cellulase to study the cell walls of *Phytophthora cinnamomi* and to form protoplasts. The amounts of cellulase they used were higher (about 0.2 unit cellulase/mg. cell wall) than in the present work. A unit of this cellulase (see Methods) approximately equals 10.8 units of Reese & Mandels (1963). Though the major component of this enzyme preparation is a cellulase, Bartnicki-Garcia & Lippmann stated that the lytic effectiveness was dependent also on the action of an endo β -D-(1 $\rightarrow$ 3) glucanase.

Lipases have been shown to aid fungal cell-wall degradation (Satamuro, Ono & Fujimoto, 1960) and in short-term incubation experiments also aid protoplast formation (Acha *et al.* 1966). It is evident from the results presented with the *Pythium* sp. PRL 2142 that the amounts of lipase are critical in protoplast formation; all the preparations tested in this study possessed some lipase activity. Removal of the incubation medium from the protoplasts lengthened their survival time, while addition of more lipase to the incubation medium shortened it. The substrate on which the lipases act at relatively low pH during protoplast formation is unknown. No wheat-germ lipase activity was detected after a 3 hr assay at pH 6.2 with olive oil as a substrate

(Table 2). However, the optimum pH value for lipase action can depend on the substrate and can be relatively low (Wills, 1965). One interesting observation is that protoplasts of the *Pythium* sp. PRL 2142 produced from mycelium grown in the presence of cholesterol are more stable than those obtained from mycelium grown without it. Any relationship between steroids and membrane stability is speculative at present. Proteases have been reported to act synergistically with glycosidases in the degradation of a *Sclerotium* sp. and yeast cell walls (Satamuro *et al.* 1960; Tabata & Terui, 1963). They are, however, not essential for the production of protoplasts from the *Pythium* sp., since a mixture of lytic preparations from *Rhizopus arrhizus* and a *Streptomyces* sp. B 814 which lacked protease (Table 2) were effective protoplast producers (Table 4). It is hoped to purify the components of the *Streptomyces* lytic enzymes and re-evaluate the protoplast-forming system.

The main components of the *Pythium* sp. PRL 2142 cell walls appear to be an unsubstituted β -D-(1 $\rightarrow$ 3)-glucan, a β -D-(1 $\rightarrow$ 6) substituted β -D-(1 $\rightarrow$ 3) glucan and cellulose, as judged from the analysis of the products of enzymic hydrolysis. They appear, therefore, to be quite similar to the walls of other oomycetous genera (Aronson *et al.* 1967; Bartnicki-Garcia & Lippman, 1967; Wang & Bartnicki-Garcia, 1966). Mitchell & Sabar (1966) reported the presence of β -D-(1 $\rightarrow$ 2) glucan in the walls of *P. butleri*, but no evidence for this glucan was found in *Pythium* sp. PRL 2142. Evidence of layering of cell-wall components has been presented by enzymic techniques (Skujins, Potgieter & Alexander, 1965; Villanueva, 1966). However, no evidence of layering of the *Pythium* walls has been found from an analysis of 'time-sequence' enzymic hydrolysates.

Several fungi (Lloyd *et al.* 1965; Potgieter & Alexander, 1966) show marked resistance to degradation by lytic preparations from other micro-organisms. Lloyd *et al.* (1965) suggested that fungal lysis in the soil may be an autolytic process, induced by toxic metabolites from antagonistic micro-organisms. The control of *Pythium de baryanum* in the soil has been achieved by the introduction of micro-organisms capable of lysing its mycelium (Mitchell & Hurwitz, 1965). The results found with *Pythium* sp. PRL 2142 indicate that heterolytic mycolysis might be the singular process active in the control of *Pythium* in the soil.

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