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The Lysing Action of Enzymes on a Sample of Mycelium of *Penicillium griseofulvum* Dierckx

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Soil organic matter is composed, at least in part, of living micro-organisms and those parts of their remains which are resistant to chemical and enzymic decomposition. A knowledge of the action of enzymes on fungal mycelium may perhaps give some idea of the composition of this resistant fraction. An examination has, therefore, been made of the breakdown by enzymic action of a sample of mycelium of *Penicillium griseofulvum* Dierckx, the composition of which has been studied by Smithies (1952).

The effect of autolysis on the composition of mould mycelium and the nature of the products liberated have been investigated (e.g. by Woolley & Peterson, 1937) usually with special regard to the protein constituent and the production of amino acids, or the yield of a particular metabolic product.

An interesting point is mentioned by Foster (1949) that, whereas in certain bacteria and actinomycetes the phenomenon of self digestion is well known, this does not occur amongst the filamentous fungi, and there remains an enzyme-resistant portion.

This paper deals with the liberation and breakdown of the carbohydrate, protein and chitin con-

stituents of the mycelium, both on autolysis of the air-dried mycelium and on treatment with enzymes from other sources.

MATERIALS AND GENERAL METHODS

Unless otherwise stated, these were as given by Smithies (1952).

Protein. This was estimated by hydrolysis of mycelium with 6N-HCl and determination of α -amino-acid N by the ninhydrin- CO_2 titration method of Van Slyke, MacFadyen & Hamilton (1941), which represents about 72% of the total protein N (Smithies, 1952).

Chitin. This was estimated in mycelium samples by determination of glucosamine hydrochloride in 6N-HCl hydrolysates (Smithies, 1952).

Carbohydrate. This was determined in mycelium by first taking up the sample in 72% (w/w) H_2SO_4 and applying the orcinol method of Pirie (1936) as used by Smithies (1952).

Snail enzyme. This was obtained from the crops and guts of starved snails (*Helix aspersa*) as described by Bawden & Pirie (1946).

Trypsin. Suspensions of dry preparations from pancreas were used as described by Holden & Tracey (1950).

Papain. This was prepared and activated as by Tracey (1948).

Enzyme 19 AP (Rohm and Haas Co., Philadelphia), preparations from *Aspergillus aureus* and *Penicillium expansum*. These were prepared as by Holden (1950).

Extraction of material soluble in organic solvents. The sample (0.2–0.5 g.) was suspended in a small filter-paper bag (acid-resistant Whatman no. 50), over refluxing ethanol (25 ml. containing two drops of 10N-HCl) for about 16 hr. and the extract, dried at 100–105°, weighed. This method gave results similar to those obtained when larger samples of mycelium were extracted with organic solvents before and after a mild acid hydrolysis, and the fat extracts combined.

N-Acetylglucosamine. This was estimated in mycelium autolysates by the method of Morgan & Elson (1934) and also by a modification of this method, whereby interference due to the presence of α -amino acids and sugars is removed by a preliminary acetylation (Smithies, 1953).

Manometric measurements. These were made in the Warburg apparatus at 25°. The volume of the reaction mixtures was 3 ml. O_2 uptakes were measured directly, with KOH in the centre cups.

Incubations. These were in general carried out at 37° in stoppered centrifuge tubes, with a drop of toluene added to maintain sterility.

EXPERIMENTS AND RESULTS

Autolysis of mycelium

These experiments were made on a batch of mycelium grown in November 1949. A portion, referred to as 'fresh mycelium', was autolysed only 7 days after harvesting during which time it was allowed to dry in air at room temperature. Further autolysis experiments on the same batch were carried out after a lapse of 2½–3 yr. This portion of the sample was powdered in a small laboratory mill and stored at room temperature in a stoppered glass jar and is subsequently referred to as 'stored mycelium'.

Autolysis of 'fresh mycelium'. The sample (50 g.) was macerated in a blender and the aqueous suspension, containing thymol crystals, made up to 500 ml. and kept at room temperature in a flask loosely plugged with cotton wool. The pH (initially 5.5) gradually increased to pH 8.0.

After 15 days a sample was removed for extraction of the residual suspended mycelium with 1% (w/v) NaOH following the procedure of Bohonos, Woolley & Peterson (1942). The extract was acidified with glacial acetic acid, and the precipitate (10 mg./g. mycelium) which formed at pH 3 collected. After purification it had the approximate composition (moisture free): N, 11.4; protein, 65; carbohydrate (as hexosan), 10; chitin, 2%. Bohonos *et al.* claimed that their protein preparation (from *A. fischeri*) was resistant to digestion by proteases.

The rest of the autolysate remained at room temperature for about 3 months, without any contamination by micro-organisms being observed. After this it was corked and stored at 0–2°. Samples were withdrawn when required.

The final chocolate-brown autolysate had a dry-matter content which accounted for only about 65% of the original solid material, the remainder having been lost as volatile products of autolysis. The composition of the autolysate and the recovery of the various components is given in Table 1. No free α -amino acids, N-acetylglucosamine or reducing sugar could be detected. The solution had a small reducing value, but that this was not due to sugar was shown by the fact that the whole of the carbohydrate was colloidal, and no sugar could be detected by paper chromatography.

Autolysis of 'stored mycelium'. The results of a typical experiment are contained in Table 2. Very little change takes place in the composition of the mycelium on storage. Analyses performed within the first 6 months after harvesting and after 3 yr. storage, showed that extracts of the mycelium made at 100° contained, in each case, only a trace of reducing sugar and N-acetylglucosamine. Ammonia accounted for 2% of the total N at both times, but the amount of free α -amino-acid N increased from about 3 to 7% of the total N. Slow hydrolysis of protein therefore occurs on storage.

Table 3 compares amounts of mycelial components dispersed by autolysis and by water at 100°, and gives some idea of the increase in extraction due to enzyme action.

Table 1. *Effect of autolysis for 3 months on the composition of mycelium which had been air-dried for 7 days only, after harvesting*

	Composition		Percentage recovery of component in autolysate (total recovery of dry matter = 65%)	Composition		Percentage of total component in autolysate found in suspended material
	Of original mycelium	Of autolysate (M_A)		Dispersed or dissolved material in autolysate (30% of total dry matter)	Suspended material in autolysate (70% of total dry matter) (M_B)	
N (mg./g. dry wt.)	39	47	78	118	19	28
P (mg./g. dry wt.)	7.2	11.8	107	31	4	24
Material soluble in organic solvents (mg./g. dry wt.)	330	270	53	540	180	46
Total carbohydrate (as hexosan) (mg./g. dry wt.)	390	480	80	320	540	80
Protein (mg./g. dry wt.)	150	90	39	200	50	39
Protein N (% of total N)	63	32	—	34	43	—
Chitin (mg./g. dry wt.)	60	50	56	23	68	89
Chitin N (% of total N)	11	7	—	1.4	25	—
NH ₃ -N (% of total N)	2	36	—	42	0	—

Table 2. *The effect of autolysis on the composition of stored mycelium*

(2.0 g. mycelium, containing 1.8 g. dry matter, incubated in 20 ml. water at 37° for 20 days. After centrifuging, the supernatant was combined with one water wash of the undispersed residue, which was air dried and powdered.)

	Composition			Percentage of total component of mycelium found in suspended material
	Stored mycelium	Suspended material in autolysate M_c (65% recovery of dry matter)	Dispersed material in autolysate (33% recovery of dry matter)	
N (mg./g. dry wt.)	39	25	65	42
P (mg./g. dry wt.)	7.2	3	17	27
Material soluble in organic solvents (mg./g. dry wt.)	330	220	Not estimated	43
Total carbohydrate (as hexosan) (mg./g. dry wt.)	390	425	390	71
Reducing sugar (as glucose) (mg./g. dry wt.)	Trace	0	180	—
Protein (mg./g. dry wt.)	150	90	Not estimated	39
Protein N (% of total N)	52	60	—	—
Free α -amino-acid N (% of total N)	7	0	45	—
Ammonia N (% of total N)	2	0	10	—
Chitin (mg./g. dry wt.)	60	65	Not estimated	70
Chitin N (% of total N)	11	18	—	—
N-Acetylglucosamine N (% of total N)	0.2	—	1.6	—

Table 3. *Increase in extraction of mycelium due to enzyme action*

	Percentage of constituent of stored mycelium dispersed	
	On autolysis	On extraction with water at 100°
Total dry matter	34	24
Nitrogen	55	37
Carbohydrate	29	12
Protein	61	15
Chitin	30	Trace only

Enzymic breakdown of mycelium constituents

Carbohydrate. The fresh wet mycelium, immediately after harvesting, had a noticeable cellulase activity, sufficient to make it difficult to dialyse extracts using cellophan membranes at room temperature. The effect was no longer apparent after air drying, and, perhaps because of this, the carbohydrate of the fresh air-dried mycelium is the most stable of the constituents analysed (Table 1). The autolysate contained no reducing sugar and the oxidases bringing about its destruction were still weakly active in the stored mycelium, which took up a small amount of O_2 when shaken in the Warburg apparatus in 0.1 M-sodium phosphate buffer (pH 7, sat. with thymol). The amount taken up was increased in the presence of glucose, but ethanolamine or DL-alanine caused no such change, showing the absence of amine oxidase or amino-acid oxidase. In a typical experiment 300 mg. of mycelium in 2 hr. took up 45 μ l. O_2 . In the presence of 0.2 ml. 0.1 M-glucose this was increased to 63 μ l. Complete oxidation of this amount of glucose to gluconic acid requires 440 μ l. O_2 . After incubation of the stored mycelium 70% of the total carbohydrate remained undispersed and the 14% obtained as reducing sugar in solution was identified as glucose by paper chromatography. Glucose only was recognized by this means in acid hydro-

lysates of the carbohydrate of M_A and M_C (Tables 1 and 2). Glucose, together with traces of galactose and mannose, was detected in acid hydrolysates of stored mycelium.

The breakdown was studied of the carbohydrate (6–8 mg./sample) of M_B and M_C by various enzymes in 0.1 M-sodium acetate buffer (pH 5) at 37°. In preliminary experiments, the hydrolysates (total vol. 2 ml.) were incubated 48 hr. and, after centrifuging, the total carbohydrate and reducing sugar in the supernatant determined. The following preparations dispersed less than 10% of the total carbohydrate (allowing for blanks carried out on the enzyme alone), and no further work was done using them: enzyme 19 AP (0.5 ml.); preparations from *A. aureus* and *P. expansum* (1 ml.); trypsin activated with NaCl (0.2 ml. at pH 7). The snail enzyme (0.5 ml.) liberated about 35% of the carbohydrate of M_B and 50% of M_C (Tables 1 and 2). In either case no further carbohydrate was dispersed after five such incubations, and the total amounts liberated were: M_B , 60% of the total carbohydrate and 108 mg. reducing sugar (as glucose)/100 mg. total dispersed carbohydrate; M_C , 85% of the total carbohydrate and 88 mg. reducing sugar (as glucose)/100 mg. total dispersed carbohydrate.

Glucose was the only sugar detected in the digests and the high values of the reducing sugar (of which less than 10% is due to N-acetylglucosamine liberated by the chitinase activity of the juice) indicate that no oligosaccharides accumulate.

The mycelial carbohydrate is not cellulose, but the efficiency of snail juice, compared with other enzyme sources, in digesting it, parallels its efficiency in breaking down cellulose.

Protein. The mycelium contains strong protease activity, which persists on storage. In the fresh mycelium, however, amino-acid oxidase is also present, amino acids do not accumulate on autolysis and the ammonia formed constitutes 36% of the total N, although, since the pH rises from 5.5 to 8, some is lost. The oxidase is quite inactive in the stored mycelium, amino acids produced by the stable protease accumulate in solution, and there is practically no change in pH.

The proteolytic enzyme was studied by incubating 40–60 mg. stored mycelium in 1 ml. water or buffer solution at 37° for 24 hr. under specified conditions. The amount of proteolysis was estimated by comparing the amount of α -amino-acid N found, with the total which would be produced on acid hydrolysis.

In sodium diethylbarbiturate-sodium acetate buffer (Michaelis, 1931), it had an optimum activity at pH 7.5–8.0; at pH 5 it was 80%, and at pH 3.5, 70% as active. The protease, in this resemblance in optimum pH to the trypsin activity of pancreas extracts, is similar to other mould proteases that have been studied (Benger, Johnson & Peterson, 1937; Dion, 1950). At the optimum pH, up to 40% of the protein was hydrolysed after 24 hr., and a maximum of 70% after a week's incubation.

The resistance of various mycelium preparations to digestion by trypsin was studied, trypsin being chosen because of its resemblance to fungal protease and the fact that Bohonos *et al.* (1942) found it to be the most effective of the enzymes tried by them in the attempted digestion of a resistant protein extract of mycelium. Furthermore, preliminary experiments on *P. griseofulvum* protein showed trypsin to be about twice as effective as activated papain. The results are given in Table 4. The protein of autolysed mycelium is more resistant than that of the original stored mycelium, although there is no significant difference between the various autolysis preparations. In particular, the dispersed protein of the fresh-mycelium autolysate, and also the alkali-extracted protein of the stored mycelium, might have been expected to show enhanced resistance to autolysis.

Table 4. *Digestion of mycelium protein with trypsin*

(The sample, containing about 0.4–0.6 mg. protein N + 1 ml. 0.1M-sodium phosphate buffer (pH 7.5) + 0.2 ml. 1% (w/v) trypsin, incubated 24 hr. at 37°.)

Preparation	α -Amino-acid N as % of total found on acid hydrolysis
Gelatin	36
*Stored mycelium	38–43
M_A †	17
M_S †	18–20
M_C †	24
Alkali-extracted protein	22–25
Dispersed protein in fresh-mycelium autolysate	23

* These samples contained 0.2–1.0 mg. protein N. The mould protease was deactivated by boiling.

† Table 1.

† Table 2.

A mycelium sample (0.4 g.) was subjected to autolysis in 5 ml. 0.1M sodium phosphate buffer (pH 7.5) for 7 days, followed by three incubations with 1 ml. trypsin solution and 4 ml. buffer, each of 2 days' duration. Subsequent incubations with trypsin, followed by activated papain at pH 5 and pepsin at pH 1–2, removed no more than traces of N. Measurements of free α -amino-acid N in the autolysate and trypsin digests showed that about 90% of the total protein had been extracted as amino acid, while acid hydrolysis indicated that the residue after water and ethanol washings

contained about 15% of the original protein content of the mycelium. 10–15% of the mycelium protein is therefore resistant to enzymic digestion. Acid hydrolysates (15 hr. in 6N-HCl at 100°) of gelatin, stored mycelium, alkali-extracted protein, M_A (Table 1), and the protease-resistant residue, after removal of volatile acid by distillation *in vacuo* and neutralization, were tested in the Warburg apparatus with a preparation of D-amino-acid oxidase from sheep kidney, using the methods given by Bender & Krebs (1950). In each case (using about 5 mg. hydrolysed protein/sample) not more than 10 μ l. O₂ was absorbed in 2 hr., whereas the addition of 1.0 mg. DL-alanine to a hydrolysate sample brought about an uptake of 60 μ l. Protease resistance of mycelium preparations is thus not due to an accumulation of D-amino-acid residues.

Chitin. Like the carbohydrase activity, chitinase activity is probably diminished on air drying. The chitin of the mycelium is thus more resistant than the protein to autolysis. There is still, however, slight chitinase activity in the stored mycelium and 10% of the chitin N was found in solution as N-acetylglucosamine, where its presence was confirmed by paper chromatography.

The digestion of mycelial chitin was studied using the digests prepared for the breakdown of carbohydrate. Enzyme 19 AP, and preparations from *A. aureus* and *P. digitatum* had feeble chitinase activity compared with snail digestive juice. After five incubations with this last, from 80 to 90% of the chitin N was dispersed as N-acetylglucosamine. Subsequent incubations brought out no more, although accurate estimations are difficult when only small amounts are in solution, owing to the relatively high apparent N-acetylglucosamine content of the snail-juice blanks (about 10 μ g. N-acetylglucosamine N/ml.). HCl hydrolysates were therefore prepared from the undigested residues and the deposit formed in the snail enzyme blank and the glucosamine hydrochloride content estimated. The amount of net residual chitin in the samples was from 3 to 8% of the original. Although these figures have no great accuracy, they confirm that only about 10%, or maybe less, of the chitin resists digestion.

DISCUSSION

The results obtained give some general idea of the breakdown of mycelium of *P. griseofulvum* by enzyme action. The lysing power of the enzyme systems of the mycelium is so considerable that a preliminary study of the effect of autolysis on the mycelial composition is required. About 55% of the fresh air-dried mycelium was dispersed in this way, including 84% of the protein, and it is very probable that, had not the carbohydrases and chitinases suffered a loss in activity through drying, there would have been a similar dispersion of carbohydrate and chitin. These components, although relatively more stable than protein to autolysis, are readily dispersed by snail digestive juice, which is known to contain a powerful cellulase and chitinase. After several incubations all but about 10% of the original chitin is dispersed and from 11 to 25% of the carbohydrate, the lower figure being obtained from data on the digestion of M_C , and the higher on the

apparently more resistant M_B (Tables 1 and 2). The reason for this discrepancy is not known. However, these figures show that about a tenth of the whole mycelium, including a part of each fraction analysed, is resistant to enzymic digestion or is not available to the enzyme. On the other hand, proteases, cellulases and chitinases are widespread in micro-organisms and invertebrates and their presence from these sources in the soil will in the absence of any stabilizing factors bring about the dispersion of the greater part of such fungal mycelium.

SUMMARY

1. Air-dried mycelium of *Penicillium griseofulvum* was subjected to autolysis. The suspended matter in the final product contained 16 % of the original protein, 64 % of the carbohydrate and 50 % of the chitin. The solution contained dispersed protein and carbohydrate, but no α -amino acids or reducing sugar.

2. Air-dried mycelium after 2½–3 yr. storage was autolysed. The suspended material contained

39 % of the original protein, 71 % of the carbohydrate and 70 % of the chitin. The solution contained amino acids, reducing sugar and *N*-acetylglucosamine.

3. The protease of the mycelium is stable to storage. Much polysaccharide-splitting and chitinase activity is lost on drying, but some of the residual activity remains after 3 yr. Such stored mycelium contains a very weak glucose oxidase, but no α -amino-acid oxidase, which was presumably very active in the fresh dry mycelium.

4. Little change takes place in the composition of the mycelium on storage except for a slight hydrolysis of protein.

5. 10–15 % of the mycelium protein resists digestion with proteases; 10–25 % of the carbohydrate and about 10 % of the chitin resists digestion with juice from the alimentary tract of snails.

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The Oxidation of Phenylacetaldehyde by Plant Saps

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Kenten & Mann (1951) found that the total oxygen uptake obtained when pea-seedling extracts catalysed the oxidation of β -phenylethylamine was in excess of that required for the oxidation of the amine to phenylacetaldehyde. This suggested the presence of an aldehyde oxidase in the extracts. In accordance with this view it was found that large increases in the oxygen uptake were produced when phenylacetaldehyde was added to pea-seedling extracts. The oxidation products were not determined, although preliminary tests suggested that

the oxidation was accompanied with hydrogen peroxide formation. By analogy with the known aldehyde oxidases phenylacetic acid was presumed to be the oxidation product. Since Skoog (1937) and Gordon & Sanchez-Nieva (1949) had obtained evidence that tryptamine could function as a precursor of 3-indolylacetic acid in plants, Kenten & Mann (1951) suggested that the successive action of amine oxidase and the aldehyde oxidase on tryptamine was a possible mechanism for the formation of 3-indolylacetic acid.