Absence of Laccase from Yellow-spored Mutants of *Aspergillus nidulans*

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SUMMARY

Green-spored strains of *Aspergillus nidulans* contain a *p*-diphenol oxidase (laccase) which is only formed during sporulation and is absent from yellow-spored mutants. The enzyme was assayed colorimetrically using *N,N*-dimethyl-*p*-phenylenediamine as substrate. Other substrates oxidized were pyrogallol, gallic acid and 2,6-dimethoxyphenol. Quinol, catechol and *p*-cresol were not detectably oxidized. Experiments with a temperature-sensitive yellow-spored mutant showed that the *ygA* locus of *A. nidulans* is a structural gene for at least a component of the enzyme, while indirect evidence suggests that the enzyme also contains copper. Yellow-green-spored mutants of the *ygA* locus produce an inactive enzyme whose activity can be restored by dialysis against copper salts. Under certain conditions the enzyme itself can diffuse from a wild-type colony to a yellow-spored mutant colony and there act to give green spore pigment. From this it is deduced that the normal site of action of the enzyme may be external to the cell membrane. A similar enzyme has also been found in conidiating cultures of *Aspergillus niger* and *Penicillium brevicompactum*.

INTRODUCTION

One of the earliest mutants used in genetic investigations of *Aspergillus nidulans* was a yellow-spored derivative of this normally green-spored species (Pontecorvo *et al.* 1953) and both green- and yellow-spored species of *Aspergillus* and *Penicillium* occur. The enzymatic basis for the difference, however, has not been investigated.

Pontecorvo *et al.* (1953) found that spore colour mutants of *Aspergillus nidulans* were autonomous in action, i.e. the colour of the spore corresponded to the genotype of the nucleus it contained. Even in a heterokaryon where one conidial head might bear conidia of different genotypes, the colour of the conidia was not influenced by others on the same head. However, when grown on a rich medium whose pH is below neutrality, some crossfeeding of yellow-spored (*y*) mutants by *y*⁺ strains occurs. The identification of the crossfeeding material as a *p*-diphenol oxidase (laccase) is reported here. This identification was suggested by the macromolecular nature of the crossfeeding material, the probable phenolic nature of the spore pigment (N. J. McCorkindale, personal communication), and the apparent dependence of green, as opposed to yellow pigmentation, on copper (Agnihotri, 1967; Holt & Macdonald, 1967), and is confirmed by the ability of cell-free extracts of the wild-type, but not yellow-spored mutants, to oxidize various phenolic substrates.

METHODS

Organisms. Strains of *Aspergillus nidulans* were from the stocks held in the Department of Genetics, The University of Glasgow (Pontecorvo *et al.* 1953; Clutterbuck, 1969*b*). The standard green-spored strain used was a biotin-requiring mutant, *biA1*, and unless otherwise stated, this was compared with strains carrying the yellow spore colour mutation *yA2*. The white-spored strain *biA1;wA9* was also frequently used for the preparation of enzyme extracts.

Mutants of *Aspergillus niger* were obtained from Dr P. Lhoas, Department of Biochemistry, Imperial College, London. A culture of *Penicillium brevicompactum* was obtained from the Botany Department, Glasgow University, and the culture of *Polyporus versicolor* from the Department of Microbiology, Queen Elizabeth College, London.

Cultures. Conidiating mycelium for enzyme extraction was grown in a special surface culture. This consisted of a vented plastic Petri dish containing approximately 20 ml of complete medium agar (Clutterbuck, 1969*a*) overlaid with 4 ml of liquid complete medium containing the inoculum of approximately 10^5 conidia/dish. This depth of medium was shallow enough to allow the mycelium to come to the surface and conidiate, but the mycelial mat so formed could still be readily peeled away from the underlying agar. The dishes were incubated at 37° for 2 days on a levelled incubator shelf.

Crude enzyme extracts. The unwashed mycelium was homogenized in an M.S.E. homogenizer (for volumes of 50 ml or more) or (for smaller volumes) ground with sand in a mortar. The homogenate was centrifuged for 5 min at 2500g and the supernatant shaken for 3 min with chloroform at room temperature, recentrifuged and degassed on a suction pump for 5 min. Typically, crude extracts contained about 4 mg of protein and 2 units (see below) of *p*-diphenol oxidase/ml, i.e. a specific activity of 0.5.

Purification and molecular weight determination. The enzyme was adsorbed batchwise on to DEAE-cellulose at pH 7.5 in 0.1 M-sodium phosphate buffer containing 0.1 M-NaCl. It was eluted on a Buchner funnel with the same buffer plus 0.5 M-NaCl. The eluate was then dialysed against 0.1 M-sodium phosphate buffer, pH 6.0, adsorbed on to a DEAE column 1.5 cm diam. $\times$ 16 cm in the same buffer and eluted with a gradient of 0 to 0.7 M-NaCl at pH 6.0. The maximum specific activity obtained by this means was 12.0.

For further purification and molecular weight determination, Sephadex G 100 or G 200 in 0.1 M-sodium phosphate buffer pH 7.0 was used in a Sephadex column 2.5 cm diam. $\times$ 36 cm with sample applicator and an L.K.B. 'Miniflow' pump giving a flow of 12.5 ml/h. Samples were added in a volume of 1.0 to 1.5 ml. For purification, extracts were concentrated both before and after passing through the column by placing in a dialysis sac in Carbowax 4000 (G. T. Gurr Ltd., Chadwell Heath, Essex).

Crossfeeding detection plates. Wells (1 cm diam.) were cut in the agar of a plate containing minimal medium (Pontecorvo *et al.* 1953) in which 15 mM-ammonium tartrate replaced sodium nitrate. The wells were inoculated with conidia of a *stuA1 yA2* strain which produced yellow conidia on very stunted conidiophores (Clutterbuck, 1969*a*) so that many of the conidia were in contact with the medium. After incubation at 37° for 24 h the enzyme extract to be tested was added to the wells. After a further 12 to 24 h incubation, enzyme activity was indicated by the presence of green spores (frequently best seen from the under-side of the plate) borne by the otherwise yellow-spored strain.

Colorimetric enzyme assay. The oxidation of *N,N*-dimethyl-*p*-phenylenediamine sulphate (DMP) was used in a colorimetric assay. The initial rate of increase in extinction at 550 nm was followed for 100 s at 37° in a Unicam SP 800 spectrophotometer in the presence and

absence of enzyme. The reaction mixture consisted of 2.5 ml buffer, pH 6.0 (37 mM-citric acid, 126 mM-disodium hydrogen phosphate) + 0.1 ml of filtered aqueous DMP (20 mg/ml) + 0.1 ml enzyme. Activities expressed as increase in extinction/100 s were multiplied by a factor of 0.2 to convert to standard units (μmol substrate oxidized/min, see Results).

Starch-gel electrophoresis. This was carried out by Poulik's (1957) modification of Smithies' (1955) system. For enzyme detection gel slices were immersed in citrate-phosphate buffer pH 6.0 containing 0.125 mg/ml DMP or a mixture of 0.125 mg/ml DMP and 0.125 mg/ml α -naphthol. Staining of *p*-diphenol oxidase bands occurred within 30 min at 37°.

RESULTS

Crossfeeding experiments. When strains are plated together on the complete medium described by Mackintosh & Pritchard (1963) a band of green conidia may be formed at the junction of adjacent white and yellow colonies. The green conidia are often somewhat yellowish, suggesting that it is genotypically *yellow* conidia that are becoming green, and this is confirmed by plating out, which gives only yellow-spored colonies. Green-spored colonies may also be able to crossfeed to yellow ones in this way, but this would be less readily detectable.

Extracts of green- or white-spored strains were prepared and fed to yellow-spored strains on separate plates. In many cases green spores were observable on the test plate. Crossfeeding is most effective when spores on the test plate are in contact with a low pH medium. Low pH and a rich (e.g. 'complete') medium also enhances production of the cross-feeding material.

The final version of this procedure (described in Methods) was used to investigate the properties of the crossfeeding material which were confirmed later when a biochemical assay was available for the material. It was concluded from these preliminary experiments that, contrary to initial expectations, the crossfeeding material was a macromolecule rather than a low-molecular-weight metabolite.

Enzymatic properties of the crossfeeding material. The possibility (see Introduction) that the crossfeeding material might be a copper-containing phenol oxidase was tested by comparing extracts of genotypically *y* and *y*⁺ strains for ability to produce coloured products from various phenolic substrates. These tests showed that pyrogallol, gallic acid and *p*-phenylenediamine were oxidized by *y*⁺ extracts, but not by *y* extracts. There was no evidence of action on catechol or *p*-cresol. However, the activity recorded colorimetrically in the oxidation of pyrogallol and gallic acid increased greatly with preincubation of the substrate in buffer before addition of the enzyme. *p*-Phenylenediamine did not suffer from this disadvantage and gave repeatable results and, furthermore, *N,N*-dimethyl-*p*-phenylenediamine behaved similarly and gave a more intense colour. It was therefore selected as substrate in the standard assay.

Stability. The crossfeeding and purification procedures showed that the enzyme was very stable at 37° and could be shaken with chloroform at room temperature without loss of activity. Considerable losses, however, resulted from precipitation by low pH or by ammonium sulphate. The enzyme could be stored frozen or at 5° at a pH between 5 and 7. DEAE purified material was treated with 0.33 mg/ml trypsin for 1 h at 37°, when the loss of activity was 25 %. On the other hand, no loss of activity resulted from incubation for 2.5 h at 37° with 0.1 mg/ml pronase. The pronase was found to be fully active in the presence of the enzyme extract when checked by the method of Rinderknecht, Geokas, Silverman & Haverback (1968).

Fig. 1. Effect of pH on the oxidation of DMP. ○, Spontaneous oxidation; ●, catalysed by crude *p*-diphenol oxidase extract from *Aspergillus nidulans*. The DMP concentration was 0.86 mM in citrate-phosphate buffer. Activity was measured colorimetrically and is expressed as μmol DMP oxidized/min/ml extract.

N,N-dimethyl-*p*-phenylenediamine (DMP) as substrate. Since DMP also oxidized spontaneously at an appreciable rate, it was necessary to use an enzyme-free blank in the reference cell. The increase in extinction at 550 nm was usually linear for about 2 min, after which it declined, but this was ample time for rate measurements in the SP 800 spectrophotometer. Removal of the sample and shaking in air either during the periods of linear or declining rise in extinction had no effect on the rate, which indicates that oxygen is not limiting with the range of DMP concentrations used.

Activities measured with DMP substrate were proportional to the amount of crude extract added over a 16-fold range. The pH optimum of the crude extract was pH 6.0 (Fig. 1).

Total oxidation of DMP using peroxidase or Polyporus enzyme (see below) showed that extinction 1.0 at 550 nm was given by the oxidation of 0.2 μmol of DMP in 2.7 ml. in a 1 cm path-length cell. The conversion factor obtained in this way agrees quite closely with that given by respirometry experiments (see below) in which oxidation of DMP was coupled to oxidation of ascorbate. This similarity would only be expected if in the ascorbate coupled system, which was measured over a period of 40 min, the decline in activity found in the photometric assay after 2 min was avoided by the removal by ascorbate of the DMP reaction product.

Eight separate estimates of the K_m were made for DMP substrate at pH 6.0 with oxygen provided by air in solution. The estimates ranged from 2.4 to 7.0 mM with the mean at 3.9 mM. There was no indication that the K_m of the crude extract differed from that of extracts partially purified with DEAE cellulose.

Other substrates. Tests on substrates other than DMP are shown in Table 1. Results show

Table 1. *Substrate specificity tests of Aspergillus nidulans p-diphenol oxidase*

Buffers: A = sodium acetate, P = sodium phosphate, CP = citrate-phosphate, TM = tris-maleic. Enzyme units are $\mu\text{mol DPM/min}$ under conditions described under Methods. (C) = crude extract, (P) = partially purified by DEAE cellulose. Activities are shown semiquantitatively, ranging from - (none) to + + + (much). In colorimetric assays using crude extracts, colour production was compared with controls containing extracts of a γ (yellow spored) mutant.

Substrate	Conditions	Buffer	Enzyme units/ml	Activity
DMP 2 mM	Respirometry	CP, pH 6.0	0.14 (P)	+ + +
Quinol 2 mM, 20 mM	.	CP, pH 6.0	1.4 (P)	-
DMP 2 mM	Ascorbate 5 mM	CP, pH 6.0	1.4 (P)	+ + + +
<i>p</i> -Phenylenediamine 2 mM	Ascorbate 5 mM	CP, pH 6.0	1.4 (P)	+
<i>o</i> -Phenylenediamine 2 mM	Ascorbate 5 mM	CP, pH 6.0	1.4 (P)	-
Quinol 2 mM	Ascorbate 5 mM	CP, pH 6.0	1.4 (P)	-
Catechol 2 mM	Ascorbate 5 mM	CP, pH 6.0	1.4 (P)	-
<i>p</i> -Cresol 2 mM	Ascorbate 5 mM	CP, pH 6.0	1.4 (P)	-
Pyrogallol 2 mM	Ascorbate 5 mM	CP, pH 6.0	1.4 (P)	-
3,4-Dihydroxyphenylalanine 2 mM	Ascorbate 5 mM	CP, pH 6.0	1.4 (P)	-
DMP 0.4-6.3 mM	Colorimetry	CP, pH 6.0	0.024 (P)	+ + + +
<i>N,N'</i> -dimethyl- <i>p</i> -phenylenediamine 0.4-6.3 mM	.	CP, pH 6.0	0.024 (P)	+ + + +
<i>p</i> -Phenylenediamine 0.4-6.3 mM	.	P, pH 6.7	0.048 (P)	+ + +
Pyrogallol 5 mM	Substrate preincubated	TM, pH 6.0-8.0	Unknown (C)	+ +
Gallic acid 5 mM	Substrate preincubated	TM, pH 7.0-8.0	Unknown (C)	+ +
Chlorogenic acid 0.54-5.4 mM	Gelatin 2.4 mg/ml or	CP, pH 6.0	0.09 (P)	-
Catechol 1.7 mM	Tween 80 0.19 %, v/v	CP, pH 6.0	0.09 (P)	-
Quinol 1.7 mM	.	CP, pH 6.0	0.09 (P)	-
Quinol 15 mM	.	A, pH 4.0, 5.0	0.22 (C)	-
Catechol 15 mM	.	P, pH 6.0, 7.0	0.22 (C)	-
<i>p</i> -Cresol 58 mM	.		0.22 (C)	-
2,6-Dimethoxyphenol 6.5 mM	.		0.22 (C)	+ +
α -Naphthol 3.5 mM	Staining of starch-gel electrophoretograms	CP, pH 6.0	.	+
α -Naphthol 3.5 mM + DMP 10 mM		CP, pH 6.0	.	+ + + +
α -Naphthol 3.5 mM + <i>o</i> -phenylenediamine 10 mM		CP, pH 6.0	.	+ +
α -Naphthol 3.5 mM + 2,6-dimethoxyphenol 13 mM		CP, pH 6.0	.	+ +
α -Naphthol 3.5 mM + 3,4-dihydroxyphenylalanine 10 mM		CP, pH 6.0	.	+ +
α -Naphthol 3.5 mM + tyrosine 10 mM		CP, pH 6.0	.	+
α -Naphthol 3.5 mM + quinol 18 mM		CP, pH 6.0	.	+
α -Naphthol 3.5 mM + catechol 18 mM		CP, pH 6.0	.	+
α -Naphthol 3.5 mM + <i>p</i> -cresol 19 mM		CP, pH 6.0	.	+
α -Naphthol 3.5 mM + chlorogenic acid 5.7 mM		CP, pH 6.0	.	+
<i>N,N'</i> -dimethyl- <i>p</i> -phenylenediamine 10 mM		CP, pH 6.0	.	+ + + +
<i>p</i> -Penylenediamine 10 mM		CP, pH 6.0	.	+ +

Table 2. *Inhibitors of Aspergillus nidulans p-diphenol oxidase*

All assays were conducted in citrate-phosphate buffer, pH 6.0, with DMP as substrate.
(P) = partially purified enzyme, (C) = crude extract.

Inhibitor	Inhibitor concn (mM)	DMP concn (mM)	Enzyme (units/ml)	Assay	Inhibition
Diethyldithiocarbamic acid	1.0	2.0	0.07 (P)	Respirometry	100 %
Diethyldithiocarbamic acid	0.1	2.0	0.07 (P)	Respirometry	21 %
Diethyldithiocarbamic acid	0.1	2.2	0.11 (P)	Colorimetry	40 %
Sodium cyanide	1.0	2.2	0.11 (P)	Colorimetry	65 %
Sodium azide	0.1	2.2	0.11 (P)	Colorimetry	74 %
2,2'-Bipyridyl	1.0	2.2	0.09 (C)	Colorimetry	0
2-Thiouric acid	0.4	0.28-1.5	0.07 (C)	Colorimetry	< 10 %
2,4-Dithiopyrimidine	0.65	0.8-9.0	0.07 (C)	Colorimetry	Competitive ($K_i = 0.44$ mM)

that only *p*-phenylenediamines, pyrogallol and gallic acid are readily oxidized, and the latter two only after preincubation of the substrate in buffer; 2,6-dimethoxyphenol was also oxidized less readily and α -naphthol and dihydroxyphenylalanine plus α -naphthol acted as stains for electrophoresis gels. Addition of Tween 80 or gelatin (Fåhræus & Ljunggren, 1961) did not increase the range of substrates oxidized, nor did the high substrate and enzyme concentrations used by Benfield, Bocks, Bromley & Brown (1964).

The K_m values for *N,N'*-dimethyl-*p*-phenylenediamine and *p*-phenylenediamine fell within the range given by DMP.

Inhibitors. Enzyme activity was effectively inhibited (Table 2) by cyanide, azide and the copper chelator diethyldithiocarbamate, but not by bipyridyl which is primarily an iron chelator. Darlington & Scazzocchio (1967) showed that 2-thioxanthine is metabolized in *Aspergillus nidulans* via thiouric acid to a compound which inhibits the transformation of conidial pigment from yellow to green. That thiouric acid is not the inhibitor is supported by the low enzyme inhibition caused by this substance. On the other hand, 2,4-dithiopyrimidine, which does appear to act directly to cause yellow conidial pigmentation (C. Scazzocchio, personal communication) is an inhibitor of DMP oxidation. With this inhibitor, activity – as measured colorimetrically – falls off more rapidly than normal, but incubation of the enzyme with the inhibitor does not increase the inhibition, so there is no evidence of irreversible inhibition. The nature of the inhibition was checked by the statistical methods of Wilkinson (1961). Both in the presence and absence of the inhibitor, V was very similar: 0.198 and 0.204 respectively but the K_p in the presence of the inhibitor was 9.50 ± 2.02 mM, which is significantly different from the K_m of 3.95 ± 0.78 . It is concluded that inhibition by 2,4-dithiopyrimidine is competitive.

Molecular weight. The molecular weight of the enzyme was determined by Sephadex gel filtration, using haemoglobin dimer (mol. wt = 33500), bovine serum albumin monomer (mol. wt = 67000), rabbit lactic dehydrogenase (mol. wt = 135000), and *Aspergillus niger* glucose oxidase (mol. wt $\times 182000$) as standards. One run with Sephadex G 100 gave a molecular weight for the *p*-diphenol oxidase of 117000, while five runs with Sephadex G 200 gave a value of 107000 ± 1020 .

Enzyme induction. The enzyme is not present in non-conidiating cultures but is naturally induced during the course of conidiation. To follow induction, mycelium was grown in a manner giving partially synchronized conidiation (Fig. 2). The enzyme is produced shortly before conidia begin to be released, although these conidia do not show visible green pig-

Fig. 2. The time of appearance of *p*-diphenol oxidase during conidiation in *Aspergillus nidulans*. ●, *p*-Diphenol oxidase units/ml extract; △, mg protein/ml extract; ○, conidia/dish. Cultures were grown in Petri dishes containing 20 ml of complete medium agar. This was overlaid with a second 4 ml layer of complete medium containing half strength agar and 10^8 conidia of the *biAr* strain/dish. This was covered with a sheet of sterile cellophane and incubated at 37° for 24 h. At this time (0 h in the figure) the cellophane was removed and incubation continued, single dishes being harvested for analysis at intervals. The mycelium in the top layer was ground with sand with the addition of 1 ml of water. After centrifugation the pellet was resuspended in a standard volume of water and the conidia counted with a haemocytometer. Protein in the supernatant was estimated by the Folin reaction (Lowry, Rosebrough, Farr & Randall, 1951) and *p*-diphenol oxidase estimated colorimetrically and expressed in units of $\mu\text{mol DMP oxidized/min}$. The approximate time of formation of stages of the conidial apparatus is shown at the top.

ment until some hours later. In *Aspergillus nidulans* neither conidiation nor enzyme production occurred in shaken liquid cultures. In *Polyporus versicolor* laccase production in shaking culture can be greatly enhanced by 2,5-xylidine or *o*-toluidine (Fähraeus, Tullander & Ljunggren, 1958). To test the effects of these substances on *A. nidulans*, parallel cultures of this fungus and *P. versicolor* were grown in shaking liquid complete medium at 22°, and 2,5-xylidine or *o*-toluidine was added at varying times from 1 to 4 days from the start of growth. Colorimetric assays using DMP substrate showed that considerable amounts of oxidase were induced in *P. versicolor* by both agents, but no oxidase was formed by *A. nidulans*.

Activities in sporulation mutants. In *Aspergillus nidulans* three genetic loci are known at which yellow or yellowish mutants may be obtained: *yellow* (*yA*) (Pontecorvo *et al.* 1953), *yellow-green* (*ygA*) (Dorn, 1967; Clutterbuck, 1968) and *chartreuse* (*chaA*) (Käfer, 1961). The *yA* locus is shown below to be the structural locus for *p*-diphenol oxidase and no activity was found in a number of *yellow* mutants tested. Mutants at the *chartreuse* and *yellow-green* loci which produce yellowish conidia had reduced amounts of *p*-diphenol oxidase and in the latter case the activity, like the spore colour, was dependent on the pH of the growth medium (Table 3).

Starch-gel electrophoresis of extracts of the wild-type gave a single fairly fast running band

Table 3. *p*-Diphenol oxidase activity in conidiation mutants of *Aspergillus nidulans*

The enzyme was assayed colorimetrically as described under Methods.
 Presence or absence of activity is indicated by + or - signs.

Mutants	Phenotype	Activity
Wild-type	Green conidia	+
<i>yA2</i> , <i>yA18</i>	Yellow conidia	-
<i>ygA6</i>	Growth at low pH: green conidia	+
	Growth at high pH: yellow conidia	-
<i>chaA3</i>	Chartreuse (grass green) conidia	(+)
<i>wA9</i>	White conidia	+
<i>wetA6</i>	White, autolysing conidia	+
<i>fwA1</i>	Fawn conidia	+
<i>abaA1</i>	No conidia: fails at (secondary?) sterigma stage	+
<i>brlA2</i>	No conidia: fails at vesicle stage	-
<i>brlA42</i>	No conidia: fails at (primary?) sterigma stage	-

when stained with DMP. By u.v. irradiation 101 yellow or yellowish mutants were obtained from the wild-type. They were tentatively classified as 4 *yellow-green*, 16 *chartreuse*, 63 *yellow* and 18 leaky *yellow* mutants. Of the *yellow* mutants, 38, including 13 of the leaky ones, were tested for complementation with *yA2* in diploids and found to be allelic to this mutant. Electrophoresis of extracts showed, in the case of the pure yellow-spored mutants, no band active against DMP, and in the case of the leaky mutants, a band in the same position as the wild-type, stained by DMP with an intensity approximately proportional to the greenness of the spores.

Other spore colour mutants: *white* (Pontecorvo *et al.* 1953), *wet-white* (Clutterbuck, 1969*a*) and *fawn* (Clutterbuck, 1965), all possessed normal *p*-diphenol oxidase activity (Table 3). It was noticed that whereas in the green-spored cultures the activity declined as the spores aged (see Fig. 2), this decline was less marked in a *white* mutant. This suggests that in the wild-type enzyme may be lost as a result of its own tanning activity, but that this does not occur in *white* mutants because of the absence of substrate.

Mutants blocked in spore development (Clutterbuck, 1969*a*) could be used to pinpoint precisely the stage at which the enzyme is naturally induced during development. The *abacus* mutants, which are believed to be blocked at a late stage in sterigma formation, possess the enzyme, but *bristle* mutants, which are blocked at stages from vesicle formation (*brlA2*) to early sterigma formation (*brlA42*), yield no enzyme activity.

Yellow-green mutants and copper. As shown in Table 3, *yellow-green* mutants have yellow spores when grown at high pH and green spores at low pH. They also differ from the wild-type in having a paler grey-brown colour of conidiophores and sterigmata (Clutterbuck, 1969*a*). In the wild-type this conidiophore pigment is darker at high pH than at low. This suggests that in *yellow-green* mutants there is competition, perhaps for some constituent such as copper which is known to occur in both enzymes (Dixon & Webb, 1964), between the tyrosinase in the conidiophores and the laccase in the conidia. The conidiophore pigment can be abolished by *ivory* (*ivo*) mutants (Clutterbuck, 1969*a*) and the *ivoA1* mutation also restores normal green pigment to *yellow-green* mutants even when grown at high pH. This suggests that the *ivoA1* mutation results in lack of production of tyrosinase and hence reduced competition for copper.

Addition of copper salts to the medium on which a *yellow-green* strain was growing did produce some shift of spore colour towards green, but only at rather high concentrations.

Table 4. *The activation by copper salts of p-diphenol oxidase activity in extracts of a yellow-green mutant of Aspergillus nidulans*

Two separate cultures of each strain were grown and extracts of each were divided into treated and control portions. The treatment consisted of dialysis for 2 h at 5° against 7×10^{-3} M-sodium phosphate buffer pH 7.5 containing 2×10^{-2} M-CuCl, followed by dialysis against two changes of the same buffer containing 7×10^{-4} M-EDTA. The controls were treated similarly but for the absence of CuCl. Activities were determined colorimetrically and are expressed as μmol of DMP oxidized/min/ml extract.

Mutant	Spore colour	Replicate	Activity	
			Treated	Control
<i>ygA6</i>	Yellow-green	1	0.70	0
		2	0.72	0
Wild-type	Green	1	1.16	0.66
		2	1.12	1.08
<i>chaA3</i>	Chartreuse	1	1.20	0.82
		2	1.08	1.00
<i>yA3</i>	Yellow	1	0	0
		2	0	0
<i>yA5</i>	Yellow	1	0	0
		2	0	0

There was also some evidence that *yellow-green* strains were slightly more resistant to toxic levels of copper salts than the wild-type.

Treatment of *Rhus* (lacquer tree) laccase with cyanide results in loss of activity which can be regained by addition of cuprous (Omura, 1961) or cupric (Tissières, 1948) salts. Dialysis of the *Aspergillus nidulans* enzyme against 10^{-2} M-sodium cyanide, followed by dialysis against two changes of buffer, reduced the activity to 27 %. However, further dialysis against 5×10^{-3} M-CuCl followed by two changes of buffer + 0.01 M-EDTA restored the activity to 130 % of the original. Table 4 shows that dialysis against cuprous chloride can also restore activity to the totally inactive extract of a *yellow-green* mutant grown at high pH. Wild-type and *chartreuse* mutant extracts are also activated to a lesser extent by this treatment, but extracts of two *yellow* mutants were unaffected. Further experiments showed that cupric chloride was as effective as cuprous chloride. The cuprous chloride solutions would have contained cupric chloride also as a result of oxidation, so it is not known whether cuprous ions are in fact effective. NaCl solutions were ineffective.

Mutants producing temperature-sensitive enzyme. Two temperature-sensitive *yellow* mutants *yA102* and *yA31*, both shown to be alleles at the *yA* locus, have yellow conidia if grown at 37° but green or greenish conidia when grown at 22°. Extracts of *yA102* were prepared from material grown at 22° and compared with extracts of the wild-type grown at the same temperature. There was, however, no clear difference either in the temperature sensitivity of the extracts or in K_m for DMP. On the other hand, extracts of the second mutant, *yA31*, were very unstable and clearly more temperature-sensitive than the wild-type. The best activity was obtained from this mutant when it was grown on a variant of complete medium containing 1 M-NaCl and buffered to pH 6.0 and 0.1 M-phosphate. In addition the usual nitrogen source – nitrate – was omitted and the Casamino acid concentration was doubled to 3 g/l. Extracts made from wild-type and *yA31* grown on this medium at 22° were purified by chloroform treatment followed by passage through a Sephadex G 200 column. Extraction and purification were done in the presence of 1 M-NaCl and 10^{-5} M-CuCl₂. Purification and concentration by Carbowax resulted in similar activities to the crude extract,

Fig. 3. Temperature decay curves for *p*-diphenol oxidase from wild-type and the temperature-sensitive mutant (*yA31*) of *Aspergillus nidulans*. Circles, wild-type; triangles, mutant. Open symbols, crude extract; closed symbols, partially purified extract. Activities were determined colorimetrically with DMP substrate.

but was accompanied by an approximately 50-fold decline in extinction at 280 nm. Purification, however, did not result in any loss of the temperature-sensitivity difference between wild-type and mutant extracts (see Fig. 3).

Plate tests for distinguishing y and y^+ strains. The properties of the enzyme described here provide two methods for distinguishing between strains carrying y and y^+ alleles when the characteristic yellow/green spore colour difference is masked by the presence of other epistatic sporulation mutants such as *white*. A biological test is obtained by inoculating the strain to be tested approximately 5 mm from any *yellow* strain on Mackintosh & Pritchard's (1963) complete medium. If the tested strain carries the y^+ allele, the *yellow* tester will produce green spores at the junction of the two colonies as a result of crossfeeding. A biochemical test can be performed by treating conidiating colonies on any medium with DMP or a mixture of DMP and α -naphthol. A convenient procedure is to add 2 ml of melted 2.5% (w/v) agar and 6 mg α -naphthol dissolved in 0.5 ml ethanol to 7.5 ml water containing 6 mg of DMP. A 2 ml portion of this solution can be injected under the agar of a plate containing the colonies to be tested. Blue staining indicates y^+ colonies.

Comparisons with other fungi. The extracellular *p*-diphenol oxidase produced by *Polyporus versicolor* was tested for activity in the plate test used for the *Aspergillus* enzyme. No green conidia were seen, however, so there is no evidence that the *Polyporus* enzyme can oxidize the *Aspergillus* pigment *in vivo*.

Conidiating surface cultures and shaken liquid cultures of *Penicillium brevicompactum* and *Aspergillus niger* were grown and extracts made from these were tested colorimetrically with DMP for *p*-diphenol oxidase activity. Both conidiating and shaken cultures of *P. brevicompactum* gave active extracts but only the extracts of conidiating cultures of *A. niger* were

active. It is therefore apparent that *A. niger* is like *Aspergillus nidulans* in that the enzyme is formed only at conidiation, while in *P. brevicompactum* it is present even in the absence of conidiation. Five spore colour mutants of *A. niger* were also tested: *w4* and *w7* (white-spored), *i20* (ivory-spored), *o* (olive-spored) and *a* (fawn-spored). All possessed *p*-diphenol oxidase activity when conidiating.

DISCUSSION

p-Diphenol oxidases have been obtained from a number of Basidiomycete fungi, of which perhaps the best known is *Polyporus versicolor* (Fåhræus *et al.* 1958). They have also been found in the Ascomycetes *Podospira anserina* (Esser, Dick & Gielen, 1964), *Glomerella cingulata* (Walker, 1968), *Penicillium spinulosum* and *Aspergillus fumigatus* (Küster & Little, 1963). In the latter two species it is postulated that the enzyme may play a part in the synthesis of certain phenolic products, while in the wood-rotting Basidiomycetes it may act in the breakdown of lignin. In contrast to this it is clear that the *Aspergillus nidulans* enzyme is essential for the production of the green spore pigment which Wright & Pateman (1970) have shown confers greater resistance to u.v. light than its yellow counterpart.

The precise nature of the natural substrate of the enzyme described here, and of the pigment of which it is a precursor, is unknown. However, *Aspergillus niger* contains a similar enzyme when sporulating, and its pigmentation is similarly dependent on copper (Mulder, 1939). The black pigment of this species has now been analysed as a perylene derivative (Barbetta, Casnati & Ricca, 1967), so it may be that the green pigment of *Aspergillus nidulans* is a similar substance.

While the natural substrate of the enzyme is as yet unknown, the substrate specificity of the enzyme indicates that it is a *p*-diphenol oxidase or 'laccase' (E.C. 1. 10. 32) although, as with other 'laccases' (Fåhræus & Ljunggren, 1961; Küster & Little, 1963; Peisach & Levine, 1965; Walker, 1968), not all possible substrates are oxidized.

The experiments with the temperature-sensitive mutant *yA31* have shown that the purifiable enzyme is also temperature-sensitive. It is therefore clear that the *yA* locus is the structural locus for at least a component of the enzyme. The copper dialysis experiments further strongly suggest that, as with other phenol oxidases, the enzyme also contains copper, and that the function of the *ygA* locus affects the availability of this element. In this respect it appears to resemble a yellow spore mutant of *Aspergillus sojae* (Takagi, 1957, Takagi & Sakaguchi, 1957). The role of the *chaA* locus, whose mutants also have yellowish conidia, is unknown, although it was apparent in the electrophoretic tests that these mutants are also partially deficient in *p*-diphenol oxidase.

The finding that crossfeeding of the enzyme can occur from one colony to another is at first sight surprising. A simple explanation is possible however: if the spore pigment is situated outside the cell membrane, then excretion of the enzyme will also be necessary. In this case, the enzyme may – given the right conditions – be able to reach and act upon pigment precursors in other conidia, without having to traverse any further membrane.

The control of this enzyme is an interesting problem which is being investigated further. At present it is apparent that it is naturally induced at the secondary sterigma stage of conidiation and never appears in *bristle* mutants, even *brlA42*, which develops as far as the primary sterigma stage. No inducing substance has been found, but the possibilities remain that the natural substrate also acts as an inducer, that the inducer is the product of a previous developmental step, or that the induction process is of a more complex nature altogether.

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