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Serological differences between the multiple amine oxidases of yeasts and comparison of the specificities of the purified enzymes from *Candida utilis* and *Pichia pastoris*

Jeffrey GREEN, Geoffrey W. HAYWOOD and Peter J. LARGE
Department of Biochemistry, University of Hull, Hull HU6 7RX, U.K.

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1. Antiserum to purified methylamine oxidase of *Candida boidinii* formed precipitin lines (with spurs) in double-diffusion tests with crude extracts of methylamine-grown cells of the following yeast species: *Candida nagoyaensis*, *Candida nemodendra*, *Hansenula minuta*, *Hansenula polymorpha* and *Pichia pinus*. No cross-reaction was observed with extracts of *Candida lipolytica*, *Candida steatolytica*, *Candida tropicalis*, *Candida utilis*, *Pichia pastoris*, *Sporobolomyces albo-rubescens*, *Sporopachydermia cereana* or *Trigonopsis variabilis*. Quantitative enzyme assays enabled the relative titre of antiserum against the various methylamine oxidases to be determined. 2. The amine oxidases from two non-cross-reacting species, *C. utilis* and *P. pastoris*, were purified to near homogeneity. 3. The methylamine oxidases, despite their serological non-similarity, showed very similar catalytic properties to methylamine oxidase from *C. boidinii*. Their heat-stability, pH optima, molecular weights, substrate specificities and sensitivity to inhibitors are reported. 4. The benzylamine oxidases of *C. utilis* and *P. pastoris* both oxidized putrescine, and the latter enzyme failed to show any cross-reaction with antibody to *C. boidinii* methylamine oxidase. Benzylamine oxidase from *C. boidinii* itself also did not cross-react with antibody to methylamine oxidase. The heat-stability, molecular weights, substrate specificities and sensitivity to inhibitors of the benzylamine/putrescine oxidases are reported. 5. The benzylamine/putrescine oxidase of *C. utilis* differed only slightly from that of *C. boidinii*. 6. Benzylamine/putrescine oxidase from *P. pastoris* differed from the *Candida* enzymes in heat-stability, subunit molecular weight and substrate specificity. In particular it catalysed the oxidation of the primary amino groups of spermine, spermidine, lysine, ornithine and 1,2-diaminoethane, which are not substrates for either of the *Candida* benzylamine oxidases that have been purified. 7. Spermine and spermidine were oxidized at both primary amino groups; in the case of spermidine this is a different specificity from that of plasma amine oxidase. 8. Under appropriate conditions, *P. pastoris* benzylamine/putrescine oxidase (which is very easy to purify) can be a useful analytical tool in measuring polyamines.

Most yeasts can use amines as sole nitrogen source for growth (Van Dijken & Bos, 1981). With primary amines this ability is due to the possession of amine oxidases with a broad substrate-specificity (Van der Walt, 1962; Yamada *et al.*, 1966). *Candida boidinii* can produce two amine oxidases during growth on primary amines. These have very similar sensitivity to inhibitors such as semicarbazide and have similar subunit molecular weights, but differ in their heat-sensitivity and substrate specificity (Haywood & Large, 1981). An examination of 22 different yeast species revealed that multiplicity of amine oxidases occurs frequently in yeasts grown on amines as nitrogen source, and implied that differences of substrate-specificity occurred (Green *et al.*, 1982). For example, extracts of yeasts grown on n-butylamine would only in a few cases catalyse the oxidation of 1,4-diaminobutane (putrescine).

Abbreviation used: ABTS, 2,2′-azinodi-(3-ethylbenzthiazoline-6-sulphonic acid).

In order to determine the extent of the differences between the various yeast species, two further approaches have been adopted. Firstly, we have studied the cross-reaction of crude extracts of a number of different yeasts with antibody to purified methylamine oxidase from *C. boidinii*. Secondly, we have purified and characterized the oxidases from two other yeast species, *Candida utilis* and *Pichia pastoris*.

These two species were selected for four reasons. Firstly, crude extracts of both organisms grown on n-butylamine were found to oxidize putrescine (Green *et al.*, 1982), whereas neither amine oxidase from *C. boidinii* can do this (Haywood & Large, 1981). Secondly, methylamine oxidase of *C. utilis* has been shown to be located in the peroxisomes (Zwart *et al.*, 1980), and it was desirable to purify this enzyme to compare it with other yeast peroxisomal enzymes (Tolbert, 1981). Thirdly, *C. utilis* and *P. pastoris* were among the organisms of which crude extracts of methylamine-grown cells failed to cross-react with antibody to purified methylamine oxidase of *C. boidinii*. Fourthly, methylamine oxidase of *P. pastoris* has a markedly different electrophoretic mobility from those of the methylamine oxidases of the two *Candida* species (J. Green, G. W. Haywood & P. J. Large, unpublished work) and thus might be expected to have other different properties.

We find that, despite the failure of the purified methylamine oxidases from *C. utilis* and *P. pastoris* to cross-react with antibody to the methylamine oxidase from *C. boidinii*, the two oxidases differ only slightly from one another and from that of *C. boidinii* in catalytic properties. The two putrescine oxidases, while having some properties in common with the benzylamine oxidase of *C. boidinii*, show significant differences. In particular, that of *P. pastoris* differs in substrate-specificity, heat-stability and subunit molecular weight. It is able to catalyse the oxidation of the primary amino groups of the polyamines spermine and spermidine, and is the first yeast amine oxidase that we have found that can do this. No cross-reaction could be detected between antibody to *C. boidinii* methylamine oxidase and any of the benzylamine/putrescine oxidases, even that of *C. boidinii*.

Materials and methods

Materials

Horseradish peroxidase (type P8125), the diammonium salt of ABTS, benzylamine, bovine γ-globulin, Freund's incomplete adjuvant and the molecular-weight-standard proteins were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Lilly 18947 and 53225 were gifts from Eli Lilly International, Indianapolis, IN, U.S.A.

The sodium salt of ABTS was prepared as described by Haywood & Large (1981). Methylamine dehydrogenase from *Pseudomonas* AM1 was purified as described by Boulton & Large (1979). 4-Aminobutyl-Sepharose 4B was prepared as described by Shaltiel (1974). *o*-Aminobenzaldehyde was synthesized as described by Albrecht *et al.* (1962). All other chemicals were obtained from Sigma Chemical Co. or Fisons, Loughborough, Leics., U.K. All amines were used in aqueous solution adjusted to pH 5.5 with HCl.

Organisms and growth conditions

Candida utilis N.C.Y.C. 321 and *Pichia pastoris* C.B.S. 704 were grown and maintained as described for *C. boidinii* by Haywood & Large (1981). Methylamine oxidase was purified from cells grown on 30mM-methylamine hydrochloride as sole nitrogen source, and benzylamine/putrescine oxidase from cells grown on 10mM-n-butylamine hydrochloride as sole nitrogen source. *Candida tropicalis* C.B.S. 94 and the other yeast strains (Green *et al.*, 1982) were grown on methylamine as nitrogen source. Former species of the genus *Torulopsis* have been transferred to the genus *Candida* as proposed by Yarrow & Meyer (1978).

Preparation of cell-free extracts

Cell-free extracts were prepared as described by Haywood & Large (1981).

Immunochemical methods

Antiserum to methylamine oxidase of *C. boidinii* was raised in a New Zealand White rabbit by the multi-site intramuscular injection of a mixture containing 3mg of purified methylamine oxidase (Haywood & Large, 1981) and 1ml of Freund's incomplete adjuvant in a total volume of 3ml. Low concentrations of antibody were detected after 30 days, whereupon a booster dose of 1mg of enzyme plus adjuvant was injected. A blood sample (6ml) was removed 7 days thereafter. After clotting, the serum was stored at −18°C. Ouchterlony double-diffusion experiments were performed on 1.5% (w/v) agarose gel containing 0.02% NaN_3. Precipitin reactions were incubated for 24h at 15°C.

Study of enzyme–antibody reaction in crude cell-free extracts

Crude cell-free extracts were diluted to a concentration of 0.15 unit of methylamine oxidase/ml, and approx. 30munits were incubated with various amounts of antiserum, adjusted to 0.2ml with 10mM-sodium phosphate buffer, pH 7.0, containing 0.85% NaCl, in small centrifuge tubes for 24h at 4°C. The tubes were then centrifuged at 10000*g* (at 4°C) for 15min to remove the immunoprecipitate, and the supernatants were assayed for residual

Table 1. *Purification of methylamine oxidase from C. utilis and P. pastoris*
In each case 25 g wet wt. of cells grown on methylamine were used. For experimental details see the text.

Step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg of protein)	Purification	Yield (%)
(*a*) Methylamine oxidase from *C. utilis*						
1. Crude extract	50	139	1110	0.125	1	100
2. 0–55%-satn.-$(NH_4)_2SO_4$ precipitate	23	113	582	0.194	1.55	81.3
3. Sepharose 6B chromatography (combined fractions)	110	127.5	201	0.580	4.64	91.7
4. 4-Aminobutyl-Sepharose 4B chromatography (combined fractions)	65	79.4	54.6	1.44	11.52	57.1
5. DEAE-Sepharose CL-6B chromatography (combined fractions)	28	41.0	11.5	3.57	28.5	29.5
6. Hydroxyapatite chromatography (combined fractions)	47	19.3	3.4	5.68	44.4	13.9
(*b*) Methylamine oxidase from *P. pastoris*						
1. Crude extract	45	77.0	144.5	0.533	1	100
2. 30–80%-satn.-$(NH_4)_2SO_4$ precipitate	12.5	70.0	121	0.578	1.10	90.9
3. Sepharose 6B chromatography (combined fractions)	102	50.0	79.6	0.628	1.20	64.9
4. DEAE-Sepharose CL-6B chromatography (combined fractions)	10	43.1	14.8	2.91	5.5	56.0
5. Hydroxyapatite chromatography (combined fractions)	25	21.8	5.0	4.36	8.2	28.3

methylamine oxidase activity by the spectrophotometric method (Haywood & Large, 1981) and compared with a control in which the extract had been incubated with 100 μl of pre-immune serum under the same conditions.

Enzyme assays

Methylamine oxidase. This was assayed by the two methods described previously (Haywood & Large, 1981). One unit of enzyme is the amount required to catalyse the formation of ABTS radical cation at a rate of 1 mM/min at 25°C.

Benzylamine/putrescine oxidase. Putrescine oxidase activity was determined by either of the two methods described for methylamine oxidase in which methylamine was replaced by putrescine dihydrochloride (1 mM). In the case of *C. utilis* n-butylamine was also used as an assay substrate. The name 'benzylamine/putrescine oxidase' is used in connection with the enzyme to emphasize its similarity to the benzylamine oxidase of *C. boidinii*, which cannot oxidize putrescine.

Conductivity measurements

These were performed as described previously (Haywood & Large, 1981).

Purification of enzymes

Purification of all four enzymes was made difficult by the high concentration of lipid in the crude extracts. The $(NH_4)_2SO_4$ and Sepharose steps in the purifications described below were employed principally to remove this lipid.

1. Methylamine oxidase from C. utilis. All steps were performed at 4°C. A typical purification is shown in Table 1(*a*).

Step 1: preparation of extract. Frozen *C. utilis* grown on methylamine as nitrogen source was suspended in 2 vol. of 20 mM-potassium phosphate buffer, pH 7.0, and disrupted as described by Haywood & Large (1981). The pellet obtained was resuspended in 1 vol. of the same buffer and passed a second time through the pressure cell. After centrifuging for 15 min at 50000 *g* the two supernatants were combined.

Step 2: $(NH_4)_2SO_4$ treatment. The combined supernatants were adjusted to 55% saturation by addition of solid $(NH_4)_2SO_4$ and centrifuged at 50000 *g* for 20 min. The precipitate was collected.

Step 3: gel filtration. The precipitate from step 2 was dissolved in a minimum volume of 20 mM-potassium phosphate buffer, pH 7.0, and applied to a column (1 m × 2.5 cm diam.) of Sepharose 6B equilibrated with the same buffer. The column was run overnight in the same buffer, and 7 ml fractions were collected.

Step 4: 4-aminobutyl-Sepharose 4B chromatography. The combined active fractions from step 3 were applied to a column (10 cm × 2 cm diam.) of 4-aminobutyl-Sepharose 4B after dilution to 10 mM-phosphate with distilled water. The column was washed with 10 mM-potassium phosphate buffer, pH 7.0. A linear gradient of 10–150 mM-

Table 2. *Purification of benzylamine/putrescine oxidase from C. utilis*
Cells grown on n-butylamine (30g wet wt.) were used. For experimental details see the text.

Step	Volume (ml)	Total activity (units) Putrescine as substrate	Total activity (units) Butylamine as substrate	Ratio butylamine/putrescine oxidation	Total protein (mg)	Specific activity (putrescine units/mg of protein)	Purification	Yield (%)
1. Crude extract	99	58.6	357	6.10	1040	0.056	1	100
2. 30–85%-satn.-$(NH_4)_2SO_4$ precipitate	19.5	54.4	317	5.82	956	0.057	1.02	92.8
3. Sepharose 6B chromatography (combined fractions)	108	52.1	216	4.14	346	0.151	2.69	88.9
4. DEAE-cellulose chromatography (combined fractions)	65	36.4	125	3.43	81	0.450	8.02	62.1
5. Hydroxyapatite chromatography (combined fractions)	34	29.3	115	3.92	15.8	1.854	33.1	50.0
6. DEAE-Sepharose CL-6B chromatography (combined fractions after concentration)	12.5	18.6	64.3	3.45	6.3	2.952	52.7	31.7

potassium phosphate buffer, pH 7.0 (in 300ml), was applied. The major peak of activity was eluted at 44mM-phosphate. The combined fractions were concentrated to about one-fifth of the original volume by using an Amicon concentration cell with a Diaflo PM 30 membrane.

Step 5: ion-exchange chromatography on DEAE-Sepharose CL-6B. The concentrated material from step 4 was diluted with distilled water to a final phosphate concentration of 10mM. The material was applied to a column (55cm × 1cm diam.) of DEAE-Sepharose CL-6B washed with 10mM-potassium phosphate buffer, pH 7.0 before elution with a linear gradient of 10–200mM-potassium phosphate buffer, pH 7.0 (in 200ml). The major peak of activity was eluted at 52mM-potassium phosphate. The peak fractions (6ml fractions) were combined and concentrated.

Step 6: hydroxyapatite chromatography. After dilution of the concentrated material from step 5 to a final phosphate concentration of 10mM it was applied to a column (10cm × 1cm diam.) of hydroxyapatite (Bio-Rad Bio-Gel HTP), washed with 10mM-potassium phosphate buffer pH 7.0, and eluted with a linear gradient of 10–200mM-potassium phosphate buffer, pH 7.0 (in 200ml), and 3ml fractions were collected. The peak fractions were combined and concentrated before the protein concentration was determined.

2. Methylamine oxidase from P. pastoris. All steps were performed at 4°C. A typical purification is shown in Table 1(*b*).

Steps 1–3. These were essentially as described above for *C. utilis*, except that the active fraction from the $(NH_4)_2SO_4$ treatment was that precipitated at between 30% and 80% saturation.

Step 4: ion-exchange chromatography on DEAE-Sepharose CL-6B. The combined active fractions from step 3 were diluted with distilled water to a final phosphate concentration of 10mM. The material was then applied to a column (55cm × 1cm diam.) of DEAE-Sepharose CL-6B and washed with 15mM-potassium phosphate buffer, pH 7.0. The enzyme was eluted with a linear gradient of 15–500mM-potassium phosphate buffer, pH 7.0 (in 500ml). The major peak of activity was eluted at 135mM-potassium phosphate. The peak fractions were combined and concentrated.

Step 5: hydroxyapatite chromatography. The concentrated fractions from step 4 were diluted with distilled water to a final phosphate concentration of 10mM. This material was then applied to a column (30cm × 1cm diam.) of hydroxyapatite equilibrated with 10mM-potassium phosphate buffer, pH 7.0. The enzyme was eluted with a linear gradient of 10–300mM-potassium phosphate buffer, pH 7.0 (in 400ml). The peak fractions were combined and concentrated before the protein concentration was determined.

3. Benzylamine/putrescine oxidase from C. utilis. All steps were performed at 4°C. A typical purification is given in Table 2. The ratio of activity with n-butylamine to activity with putrescine (Table 2) fell as methylamine oxidase was separated from benzylamine/putrescine oxidase and subsequently remained constant throughout the purification.

Steps 1–3. These were essentially as described for methylamine oxidase from *C. utilis*, except that the

Table 3. *Purification of benzylamine/putrescine oxidase from P. pastoris*
Cells grown on n-butylamine (20g wet wt.) were used. For experimental details see the text.

Step	Volume (ml)	Total activity (units) (putrescine as substrate)	Total protein (mg)	Specific activity (units/mg of protein)	Purification	Yield (%)
1. Crude extract	47	105	322	0.326	1	100
2. 30–80%-satn.-$(NH_4)_2SO_4$ precipitate	9.5	86.2	238	0.363	1.11	82.1
3. Sepharose 6B chromatography (combined fractions)	36	54.5	20.9	2.61	8.0	51.9
4. DEAE-Sepharose CL-6B (1 M-phosphate eluate)	32.5	48.4	10.1	4.80	14.7	46.1

active fraction from the $(NH_4)_2SO_4$ treatment was that precipitated at between 30% and 85% saturation.

Step 4: ion-exchange chromatography on DEAE-cellulose. The material from step 3 was applied to a column (10cm × 1cm diam.) of Whatman DE-52 DEAE-cellulose equilibrated with 10mM-potassium phosphate buffer, pH 7.0, and eluted with a linear gradient of 10–250mM-potassium phosphate buffer, pH 7.0 (in 300ml). The peak fractions were combined and concentrated.

Step 5: hydroxyapatite chromatography. After dilution of the concentrated material from step 4 with distilled water to a final phosphate concentration of 10mM, it was applied to a column (15cm × 1cm diam.) of hydroxyapatite. After a washing with 10mM-potassium phosphate buffer, pH 7.0, the enzyme was eluted with a linear gradient of 10–200mM-potassium phosphate buffer, pH 7.0 (in 200ml). The peak fractions were combined and concentrated.

Step 6: ion-exchange chromatography on DEAE-Sepharose CL-6B. The material from step 5 was diluted with distilled water to 10mM-phosphate and applied to a column (30cm × 1cm diam.) of DEAE-Sepharose CL-6B, equilibrated with 10mM-potassium phosphate buffer, pH 7.0. After a washing with the same buffer the enzyme was eluted with a linear gradient of 10–200mM-potassium phosphate buffer, pH 7.0 (in 200ml). Fractions containing more than 50% of the maximum activity were combined and concentrated before the protein concentration was determined.

4. Benzylamine/putrescine oxidase from P. pastoris. All steps were performed at 4°C. A typical purification is shown in Table 3.

Steps 1–3. These were essentially as described above for methylamine oxidase from *C. utilis*, except that the active fraction from the $(NH_4)_2SO_4$ treatment was that precipitated at between 30% and 80% saturation.

Step 4: ion-exchange chromatography on DEAE-Sepharose CL-6B. The combined active fractions from step 3 were applied to a column (40cm × 2cm diam.) of DEAE-Sepharose CL-6B after the phosphate concentration was adjusted to 250mM with 1M-potassium phosphate buffer, pH 7.0. The column was washed with 250mM-potassium phosphate buffer, pH 7.0, before elution with 1M-potassium phosphate buffer, pH 7.0. The peak fractions were combined and concentrated before the protein concentration was determined.

Chemical determinations

These were as described previously (Haywood & Large, 1981) with the following additions. Spermine and putrescine were determined with fluorescamine (De Bernardo *et al.*, 1974) and their aldehyde oxidation products with 3-methyl-2-benzothiazolone hydrazone (Sawicki *et al.*, 1961), by using a millimolar absorption coefficient of $6.25\,mM^{-1}\cdot cm^{-1}$ at 660nm for each aldehyde group formed (Bachrach & Reches, 1966). 1-Pyrroline was determined with *o*-aminobenzaldehyde by using a millimolar absorption coefficient of $1.86\,mM^{-1}\cdot cm^{-1}$ (Bachrach & Oser, 1963).

Polyacrylamide-gel electrophoresis

This was performed as described by Haywood & Large (1981).

Stoicheiometry of methylamine and benzylamine oxidation

The stoicheiometries of methylamine and benzylamine oxidation were determined as described previously (Haywood & Large, 1981).

Stoicheiometry of P. pastoris benzylamine/putrescine oxidase with benzylamine, spermine and putrescine

This was performed essentially as in the polarographic method described by Haywood & Large (1981), except that 0.4μmol of spermine or benzylamine or 0.5μmol of putrescine was used as substrate. The reaction was allowed to proceed to

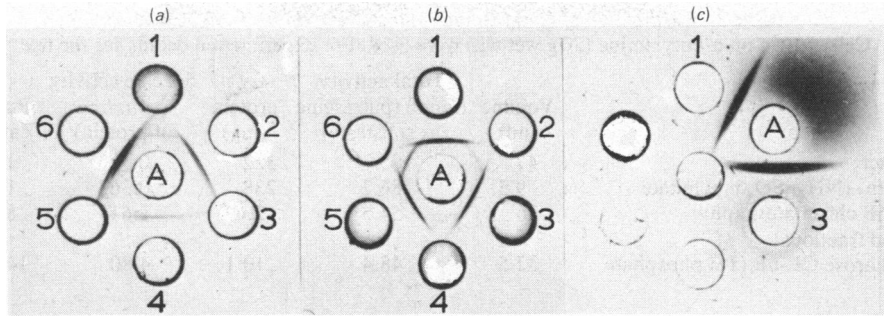


Fig. 1. *Double-immunodiffusion patterns for methylamine oxidases from different yeast species*
Well A of each plate contained antiserum (10 μl) prepared against purified methylamine oxidase of *C. boidinii*. (*a*) Each outer well contained crude extract of various yeast species containing methylamine oxidase: 1, *H. polymorpha*, 1 munit; 2, *C. nagoyaensis*, 7.8 munits; 3, *Trigonopsis variabilis*, 9 munits; 4, *P. pinus*, 5.4 munits; 5, *Sporopachydermia cereana*, 27.4 munits; 6, *C. boidinii*, 7.7 munits. (*b*) Each outer well contained crude extracts as in (*a*): 1, 3 and 5, *C. boidinii*, 8.9 munits each; 2, *P. pinus*, 4 munits; 4, *H. polymorpha*, 0.9 munit; 6, *C. nagoyaensis*, 8.4 munits. (*c*) Well no. 1 contained 8.4 munits of purified methylamine oxidase from *C. boidinii* (Haywood & Large, 1981); well no. 3 contained 16.8 munits. This plate was incubated for 24 h at 4°C. After the position of the precipitin lines had been noted, the plate was immersed in an activity stain containing (in 6.5 ml) 15 mg of 3,3'-diaminobenzidine, 400 μmol of potassium phosphate, pH 7.0, 1.5 mmol of sodium acetate, 30 μmol of methylamine hydrochloride and 600 μg of horseradish peroxidase. After 1 h, the precipitin lines had stained dark brown. The faint haloes round wells 1 and 3 are brown colour due to activity of unprecipitated enzyme. The large halo on the periphery is brown colour possibly due to catalase activity in the serum (in which some haemolysis was evident).

completion in the absence of catalase, which was then added to the reaction vessel to determine the amount of H_2O_2 produced.

Results

Serological tests on crude cell-free extracts

When antiserum to purified methylamine oxidase from *C. boidinii* was tested in double-diffusion plates with crude extracts (each containing approx. 5–20 munits of methylamine oxidase activity) of various yeast strains grown on methylamine as nitrogen source, precipitin lines were observed with some yeast strains but not others (a few examples are shown in Fig. 1*a*). No precipitin lines were observed with amine oxidase-containing extracts of *Candida lipolytica*, *Candida steatolytica*, *Candida tropicalis*, *Candida utilis*, *Pichia pastoris*, *Sporobolomyces albo-rubescens*, *Sporopachydermia cereana* and *Trigonopsis variabilis*.

Those extracts that gave positive precipitin bands were tested for immunological relatedness. Spurs were observed with extracts of the following organisms: *Candida nagoyaensis*, *Candida nemodendra*, *Hansenula minuta*, *Hansenula polymorpha* and *Pichia pinus*. Some examples are shown in Fig. 1(*b*). The precipitin line from *H. polymorpha* was so faint that it fails to show up in Fig. 1(*b*). This is due to the low protein (and antigen) concentration (see the legend to Fig. 1). Attempts to obtain a more concentrated extract failed because of well-recognized problems in breaking the cells of this particular organism. On application of the diaminobenzidine activity stain described by Haywood & Large (1981), the precipitin bands stained positively, showing that the immunoprecipitate retained enzyme activity (Fig. 1*c*).

Quantitative measurements of cross-reactivity were made by incubating various dilutions of antiserum with a fixed amount of enzyme activity (30 munits) in crude extracts of some of the species that showed cross-reaction. After 24 h at 4°C, the immunoprecipitate was removed by centrifuging, and the supernatant was assayed for residual enzyme activity. None of the cross-reacting enzymes was as effectively precipitated from crude extracts as that of *C. boidinii* (Fig. 2). From logarithmic plots of the data in Fig. 2 it was possible to calculate the amount of antiserum per unit of enzyme required to precipitate 50% of the activity. This varied from 0.33 ml of antiserum/unit of enzyme for the antigenic methylamine oxidase of *C. boidinii* to 4.67 ml of antiserum/unit for the *P. pinus* enzyme. The corresponding I_{50} values for the remaining extracts were 1.33 ml of antiserum/unit for *C. nagoyaensis* enzyme, 1.68 ml of antiserum/unit for the *C. nemodendra* enzyme and 3.47 ml of antiserum/unit for the *H. polymorpha* enzyme.

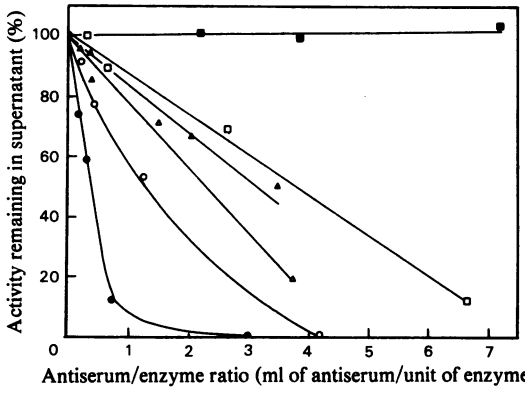


Fig. 2. *Precipitation of methylamine oxidase activity from crude extracts of various yeast species by antiserum to methylamine oxidase of C. boidinii*

Various volumes of antiserum and crude extracts of the different yeasts (all grown on methylamine as sole nitrogen source), diluted to give approx. 30munits of methylamine oxidase activity, were incubated in 10mM-sodium phosphate buffer, pH7.0, containing 0.85% NaCl, in small centrifuge tubes for 24h at 4°C. The immunoprecipitate was then centrifuged off and the supernatant was assayed for residual enzyme activity, and the results are expressed as percentages of a control that had been incubated under the same conditions with 100µl of pre-immune serum. ●, *C. boidinii*; ○, *C. nagoyaensis*; △, *C. nemodendra*; □, *P. pinus*; ▲, *H. polymorpha*; ■, *C. tropicalis* (known from immunodiffusion tests not to cross-react). Loss of enzyme activity in the controls was less than 10%.

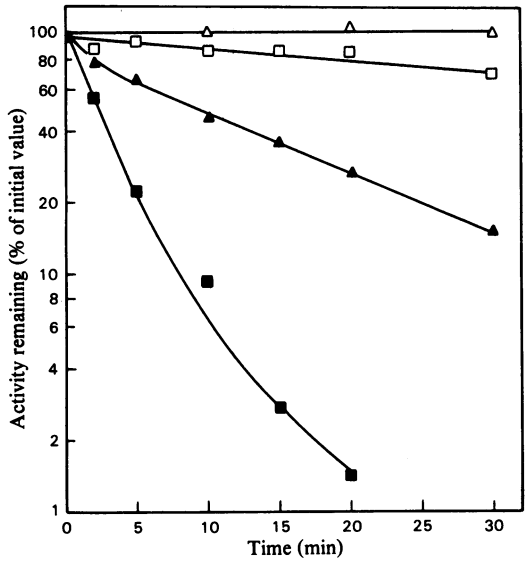


Fig. 3. *Effect of heat on purified methylamine oxidases and benzylamine/putrescine oxidases from C. utilis and P. pastoris*

Purified enzymes (0.1mg of protein/ml) were incubated at 45°C, and samples were removed at intervals into ice and assayed for residual enzyme activity. ■, Methylamine oxidase, and □, benzylamine/putrescine oxidase from *C. utilis*; ▲, methylamine oxidase, and △, benzylamine/putrescine oxidase from *P. pastoris*.

Purification of the amine oxidases from C. utilis and P. pastoris

Both methylamine oxidase and benzylamine/putrescine oxidase from *C. utilis* were difficult to purify. After the procedures described in Tables 1 and 2, both enzymes still contained traces of impurities, as assessed by polyacrylamide-gel electrophoresis, although the single active band on polyacrylamide-gel was estimated visually to comprise more than 90% of the protein present. The impurities did not interfere with the determination of subunit molecular weight.

The enzymes from *P. pastoris* were relatively easy to purify. The methylamine oxidase contained two very faint bands on polyacrylamide-gel electrophoresis due to minor impurities, and the benzylamine/putrescine oxidase one faint band. A simpler purification method than that described in Table 3 has more recently been devised, which produces the *P. pastoris* benzylamine/putrescine oxidase virtually homogeneous in a single step. This simpler method involves breaking the cells in 0.2M-potassium phosphate buffer, pH7.0. This extract is then applied direct to a column of DEAE-Sepharose CL-6B equilibrated with 0.2M-potassium phosphate buffer, pH7.0, and eluted with a linear gradient of 0.2–1.0M-potassium phosphate buffer, pH7.0. The enzyme is eluted very sharply with little or no impurities at a phosphate concentration of 0.39M.

The relative mobilities of the active bands of the four enzymes in the gel system decribed by Davis (1964) were as follows (Bromophenol Blue = 1.0): methylamine oxidase from *C. utilis*, 0.10; benzylamine/putrescine oxidase from *C. utilis*, 0.28; methylamine oxidase from *P. pastoris*, 0.61 (major band) and 0.31 (minor band); benzylamine/putrescine oxidase from *P. pastoris*, 0.36 (with faint active bands at 0.17 and 0.40).

Heat-stability and pH optima

At 45°C it was relatively easy to distinguish the four enzymes by their rates of thermal inactivation (Fig. 3). The half-lives of the four purified enzymes (protein concentration 0.1mg/ml in each case) were respectively 2.2min for methylamine oxidase from *C. utilis*, 9min for methylamine oxidase from *P. pastoris* and 64min for the benzylamine/putrescine

oxidase from *C. utilis*, and the corresponding enzyme from *P. pastoris* lost no activity during the time of the experiment (30 min).

Small differences in pH optima were also observed, but they may not be of significance. For the oxidation of methylamine, the *C. utilis* enzyme was maximally active at pH 7.5, and the optimum curve for *P. pastoris* was sharper with a maximum at pH 7.4. The benzylamine/putrescine oxidase from *C. utilis* had an optimum at pH 7.5 with benzylamine as substrate, and the *P. pastoris* enzyme showed maximum activity at pH 7.0.

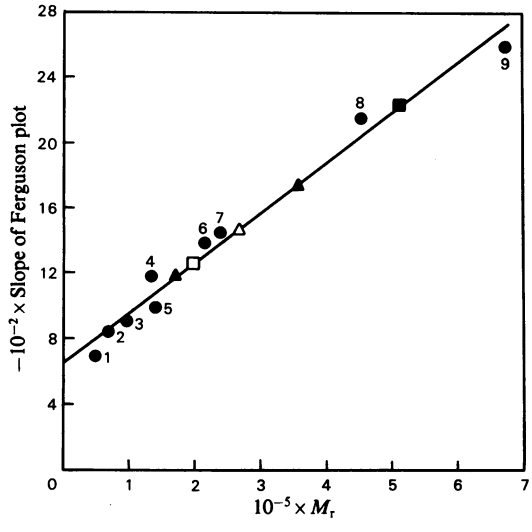


Fig. 4. *Relationship between retardation coefficient and relative molecular mass for the amine oxidases compared with various protein standards*

The retardation coefficient ($-10^2 \times$ slope of a plot of $\log[100 \times R_m]$ against acrylamide concentration) was plotted against relative molecular mass for the following standards: 1, hexokinase (yeast) (monomer); 2, bovine serum albumin (monomer); 3, hexokinase (dimer); 4, bovine serum albumin (dimer); 5, lactate dehydrogenase (rabbit muscle); 6, β-amylase (sweet-potato); 7, α-urease (Hedrick & Smith, 1968); 8, apoferritin monomer; 9, bovine thyroglobulin. ■, Methylamine oxidase, and □, benzylamine/putrescine oxidase from *C. utilis*; ▲, methylamine oxidase (two bands), and △, benzylamine/putrescine oxidase from *P. pastoris*.

Molecular weights

The relative molecular masses of the undenatured enzymes were determined by polyacrylamide-gel electrophoresis by the method of Hedrick & Smith (1968). Ferguson plots were used to determine the retardation coefficients. Retardation coefficients for standard proteins of known molecular weight were also determined under the same conditions. The retardation coefficient was then plotted against the molecular weight (Fig. 4), and hence the molecular weights in Table 4 were obtained.

The subunit molecular weights (Table 4) were determined by comparing the relative mobility of the Coomassie Blue-stained subunit bands with that of standard markers in sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Substrate specificity of the methylamine oxidases

Apparent K_m and $V_{max.}$ values were determined for a range of substrates at an oxygen concentration of 0.25 mM. These were of a similar order to the previously published values for the methylamine oxidase of *C. boidinii* (Haywood & Large, 1981), except that the enzyme from *C. utilis* showed no measurable activity with 1-aminoalkanes of chain length greater than C_6. Fig. 5(*a*) shows the ratio $V_{max.}/K_m^{app.}$ plotted as a function of carbon chain length for the 1-aminoalkanes, and it is clear that the two enzymes and that of *C. boidinii* are very similar even to the position of maxima and minima. When αω-diaminoalkanes were tested as substrates, 1,2-diaminoethane showed low activity, but no others were active until a chain length of C_9 was reached, when methylamine oxidase from *P. pastoris* showed measurable activity (Fig. 5*c*). Ethanolamine, 3-methylbutylamine and phenethylamine were also substrates. Benzylamine, histamine, spermine, spermidine and all the other substrates recorded by

Table 4. *Relative molecular masses of the amine oxidases from C. utilis and P. pastoris*

Enzyme and organism	$10^{-3} \times M_r$ (from Ferguson plots)	$10^{-3} \times$ Subunit M_r (from sodium dodecyl sulphate/polyacrylamide-gel electrophoresis)	Apparent number of subunits (ratio of the two preceding columns)
Methylamine oxidase			
C. utilis	510	85	6.0
P. pastoris	168 (major)	80	2.1
	355 (minor)		4.5
Benzylamine/putrescine oxidase			
C. utilis	190	76	2.5
P. pastoris	265	116	2.3

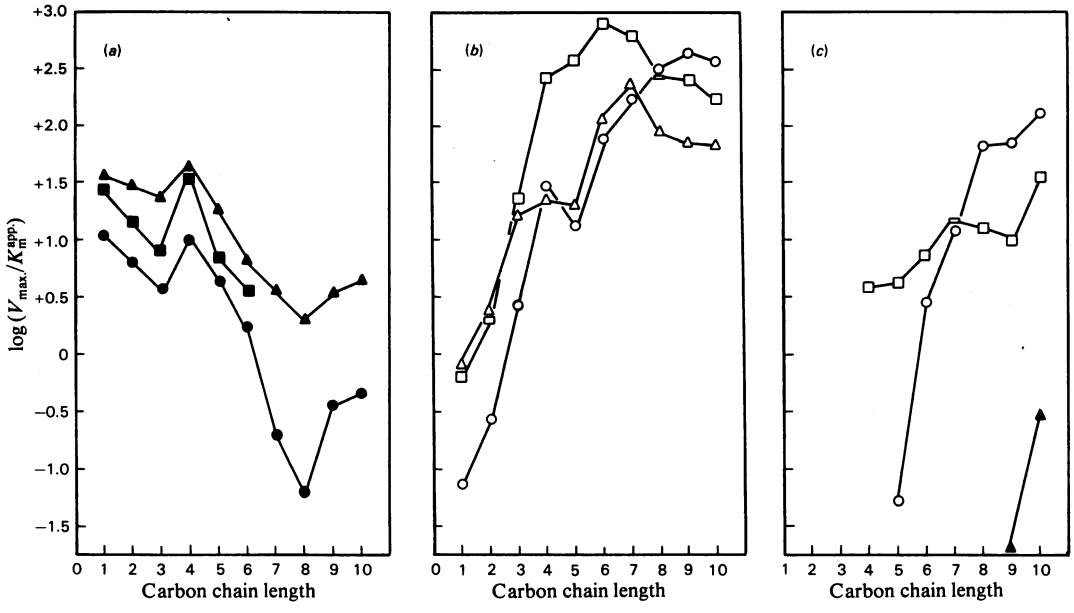


Fig. 5. *Relationship between log $V_{max.}/K_m^{app.}$ and carbon chain length of substrate for the amine oxidase of various yeast species*

The ratio $V_{max.}/K_m^{app.}$ is a measure of the affinity of the enzymes for particular substrates. (*a*) Plot of log $V_{max.}/K_m^{app.}$ for the methylamine oxidases of *C. boidinii* (●), *C. utilis* (■) and *P. pastoris* (▲) against the carbon chain length of 1-aminoalkanes. (*b*) A similar plot with the same substrates for the benzylamine/(putrescine) oxidases of *C. boidinii* (○), *C. utilis* (□) and *P. pastoris* (△). (*c*) Plot of log $V_{max.}/K_m^{app.}$ against carbon chain length for the oxidation of αω-diaminoalkanes: ○, benzylamine oxidase of *C. boidinii*; □, benzylamine/putrescine oxidase of *C. utilis*; ▲, methylamine oxidase of *P. pastoris*.

Haywood & Large (1981) as not being oxidized by the *C. boidinii* enzyme were also not substrates for the methylamine oxidases from *C. utilis* and *P. pastoris*.

Substrate-specificity of the benzylamine/putrescine oxidases

The substrate-specificities for 1-aminoalkanes of these enzymes from *C. utilis* and *P. pastoris* resembled that of *C. boidinii* benzylamine oxidase, although in most cases the ratio $V_{max.}/K_m^{app.}$ was somewhat higher (Fig. 5*b*). The chain length for maximum activity however differed in each case, being C_6 for the benzylamine/putrescine oxidase from *C. utilis*, C_7 for the enzyme from *P. pastoris* and C_9 for the *C. boidinii* enzyme. More significant differences were evident with αω-diaminoalkanes as substrates. Whereas putrescine and 1,3-diaminopropane were not substrates for benzylamine oxidase from *C. boidinii* (Haywood & Large, 1981), both were oxidized by the enzymes from *C. utilis* and *P. pastoris*. Longer-chain diamines were substrates for the benzylamine oxidases from both *Candida* species, though differences in the $V_{max.}/K_m^{app.}$ ratios were apparent (Fig. 5*c*, which shows also data for *C. boidinii* that have not been previously published). Accurate determination of $K_m^{app.}$ values for αω-diaminoalkanes for the *P. pastoris* enzyme was not possible, because they were so low (<1 μM, giving $V_{max.}/K_m^{app.} \sim 10^4$). This makes a clear distinction between the *Pichia* benzylamine/putrescine oxidase and the *Candida* enzymes. A number of other differences in substrate-specificity were found between the *P. pastoris* enzyme and the benzylamine oxidases from the two *Candida* species. The *P. pastoris* enzyme oxidized lysine, ornithine, spermine, spermidine and 1,2-diaminoethane, which were not oxidized by benzylamine oxidase from either *Candida* species. All the enzymes oxidized histamine, tyramine, ethanolamine, 3-methylbutylamine, phenethylamine, isobutylamine and agmatine. Apart from the exceptions noted above, the substrates listed as inactive with the *C. boidinii* enzyme by Haywood & Large (1981) were also not substrates for benzylamine/putrescine oxidase from either *C. utilis* or *P. pastoris*.

Sensitivity to inhibitors

All four enzymes showed essentially the same sensitivity to carbonyl reagents, chelating agents,

substrate analogues, 'amine oxidase inhibitors' and mercurials at the same concentrations as was observed with the two oxidases from *C. boidinii* (Haywood & Large, 1981).

Serological properties

Neither of the two purified methylamine oxidases cross-reacted with antibody to *C. boidinii* methylamine oxidase, despite their similarities in catalytic properties. Benzylamine/putrescine oxidase from *P. pastoris* also failed to cross-react. Benzylamine/putrescine oxidase from *C. utilis* was not available for immunological testing, but it seems probable that no cross-reaction would have been observed because benzylamine oxidase from *C. boidinii* itself failed to cross react with anti-(methylamine oxidase) serum.

Stoicheiometry of substrate oxidation

Methylamine oxidation by the two methylamine oxidases (Table 5) showed the same stoicheiometry as with *C. boidinii*. The same was true for benzylamine oxidation. The stoicheiometry for the oxidation of spermine (Table 5) is that shown in eqn. (4) below. One important fact to note was that, as previously observed with the enzymes from *C. boidinii* (Haywood & Large, 1981) the purified enzymes from *C. utilis* contained traces of catalase (although catalase was not detectable by spectrophotometric assay). The *P. pastoris* benzylamine/putrescine oxidase in contrast was totally free of catalase and gave good results when catalase was omitted from the oxygen-electrode reaction mixture (Table 5*b*, columns 3–5).

P. pastoris benzylamine/putrescine oxidase as a polyamine oxidase

Since spermine and spermidine are very good substrates for the benzylamine/putrescine oxidase of *P. pastoris* ($K_m^{app.} < 1\,\mu M$), it was important to determine the stoicheiometry of the oxidation of compounds containing more than one primary amino group. With simple $\alpha\omega$-diaminoalkanes, when

Table 5. *Determination of the stoicheiometry of the reactions catalysed by the four amine oxidases*

Substrate was incubated at 25°C with enzyme in either the chamber of an oxygen electrode or in a spectrophotometer cuvette. When reaction was complete 0.3 ml of 2.5 M-$HClO_4$ was added to stop the reaction. After neutralization and centrifuging, reactants and products were determined by the following methods: methylamine, Large *et al.* (1969); formaldehyde, Nash (1953); ammonia, Chaney & Marbach (1962); benzylamine, Obata *et al.* (1971); benzaldehyde, by absorbance measurements at 250 nm. For spermine, putrescine, aldehyde and 1-pyrroline determinations, see the text. For full experimental details see Haywood & Large (1981).

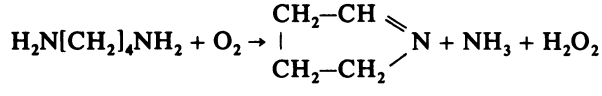
(*a*) Methylamine oxidases

	Stoicheiometry (μmol)	
	C. utilis enzyme	*P. pastoris* enzyme
Polarographic experiment		
Methylamine consumed	0.376	0.384
O_2 consumed	0.190	0.185
Formaldehyde formed	0.385	0.388
Ammonia formed	0.320	0.360
Spectrophotometric experiment		
Methylamine consumed	0.193	0.190
H_2O_2 formed	0.184	0.181

(*b*) Benzylamine/putrescine oxidases

	Stoicheiometry (μmol)				
	C. utilis enzyme		*P. pastoris* enzyme		
	Polarographic experiment	Spectrophotometric experiment	Polarographic experiments		
Benzylamine consumed	0.44	0.25	—	0.42	—
Oxygen consumed	0.20	—	0.71	0.47	0.60
Benzaldehyde formed	0.42	0.31	—	0.41	—
Ammonia formed	0.41	0.24	0.70	0.49	0.71
H_2O_2 formed	—	0.27	0.71	0.47	0.58
Spermine consumed			0.34	—	—
Aldehyde groups formed			0.61	—	—
Putrescine consumed			—	—	0.58
1-Pyrroline formed			—	—	0.28

a limiting amount of substrate was tested in the spectrophotometric assay it was clear that with 1,4-diaminobutane (putrescine) only a single amino group was oxidized, whereas with 1,8-diaminooctane both groups were oxidized although the rate of oxidation slowed down significantly after an amount of ABTS radical cation had been formed equivalent to half the total amino groups present. The molar ratio of ABTS radical cation formed to diamine oxidized was measured, and the following values were obtained: putrescine, 2.18; spermine, 3.98; spermidine, 4.4; this is in keeping with a stoicheiometry in which for spermine and spermidine two amino groups were oxidized and two molecules of H_2O_2 formed (since ABTS is a single-electron donor). To confirm this, the number of aldehyde groups formed was measured by the 3-methyl-2-benzothiazolone hydrazone method of Sawicki *et al.* (1961). The ratio of mol of aldehyde groups formed/mol of diamine oxidized was 2.08 for spermine, 2.24 for spermidine, 0.78 for 1,4-diaminobutane (putrescine) and 2.11 for 1,5-diaminopentane (cadaverine). The reason for the anomalous behaviour of putrescine may be the tendency of the mono-oxidation product 4-aminobutyraldehyde to cyclize to 1-pyrroline. Accordingly, the formation of 1-pyrroline was measured by using *o*-aminobenzaldehyde (Bachrach & Oser, 1963). No yellow colour was observed with the oxidation products from spermine, spermidine or butylamine, but a yellow colour denoted the formation of 0.89 mol of 1-pyrroline/mol of putrescine oxidized and 0.54 mol of 2,3,4,5-tetrahydropyridine/mol of cadaverine. Clearly with these two substrates, the nature of the product formed is affected by whether or not peroxidase and ABTS are present. The data thus suggest that the benzylamine/putrescine oxidase of *P. pastoris* catalyses the oxidation of both primary amino groups in all di- and poly-amines except putrescine (eqns. 1–4), and does not attack secondary amino groups.



$$H_2N[CH_2]_4NH_2 + O_2 \rightarrow \begin{matrix} CH_2{-}CH \\ | \quad\quad \diagdown \\ CH_2{-}CH_2 \quad N \end{matrix} + NH_3 + H_2O_2 \quad (1)$$

$$H_2N[CH_2]_8NH_2 + 2O_2 + 2H_2O \rightarrow OHC[CH_2]_6CHO + 2NH_3 + 2H_2O_2 \quad (2)$$

$$H_2N[CH_2]_3NH[CH_2]_4NH_2 + 2O_2 + 2H_2O \rightarrow OHC[CH_2]_2NH[CH_2]_3CHO + 2NH_3 + 2H_2O_2 \quad (3)$$

$$H_2N[CH_2]_3NH[CH_2]_4NH[CH_2]_3NH_2 + 2O_2 + 2H_2O \rightarrow OHC[CH_2]_2NH[CH_2]_4NH[CH_2]_2CHO + 2NH_3 + 2H_2O_2 \quad (4)$$

The specificity with which spermine is oxidized (eqn. 4) resembles that of bovine plasma oxidase (Tabor *et al.*, 1964) (mechanism 6 of Smith, 1972). For spermidine, however, the mechanism postulated in eqn. (3) differs from that for any enzyme so far described (Morgan, 1980), since 2 mol of aldehyde groups is formed/mol of spermidine oxidized. The failure to detect colour with *o*-aminobenzaldehyde rules out any mechanism in which putrescine is formed as an intermediate via cleavage of the secondary amino group, and then further oxidized.

Discussion

The results from the serological experiments suggest that the methylamine oxidases of yeasts are not all closely similar. Although methylamine oxidase in crude extracts of *C. nagoyaensis*, *C. nemodendra*, *H. minuta*, *H. polymorpha* and *P. pinus* cross-reacted immunologically with antiserum to methylamine oxidase of *C. boidinii*, the enzymes from *C. lipolytica*, *C. steatolytica*, *C. tropicalis*, *C. utilis*, *P. pastoris*, *Sporobolomyces albo-rubescens*, *Sporopachydermia cereana* and *Trigonopsis variabilis* showed no cross-reactivity. The observation of spurs with those extracts which showed cross-reaction shows that the enzymes are not immunologically identical (Crowle, 1961). The results with the immunodiffusion plates were borne out by the more quantitative results from the titration experiment (Fig. 2). The fact that the yeasts with cross-reacting methylamine oxidases are all methylotrophic yeasts is probably coincidental, because *P. pastoris* in the non-cross-reacting category is also a methylotroph. It is also noteworthy that the immunological differences cut across generic boundaries.

Purification of two of the methylamine oxidases that were serologically distinct from that of *C. boidinii*, namely those from *C. utilis* and *P. pastoris*, revealed that the two enzymes differed only slightly in catalytic properties from the methylamine oxidases of *C. boidinii* (Haywood & Large, 1981) and *Trichosporon* sp. (Yamada *et al.*, 1966). They both had similar subunit molecular weights, though the enzyme from *P. pastoris* showed a greater heat-stability. The *C. utilis* enzyme is known to be located in the peroxisomes (Zwart *et al.*, 1980). The methylamine oxidases in general seem to be sig-

nificantly less stable to heat than are the benzylamine/putrescine oxidases (Fig. 3). The molecular weight of the enzyme from *C. utilis* is similar to that of *C. boidinii*, whereas that of methylamine oxidase from *P. pastoris* is smaller. This, however, may not be very significant (see the Discussion section in Haywood & Large, 1981).

It is necessary to offer some explanation as to why two methylamine oxidases with closely similar catalytic properties fail to cross-react with antibody to a third, apparently closely similar, enzyme. Since the antibody to *C. boidinii* methylamine oxidase fails to inhibit the enzyme (Fig. 1*c*), it seems likely that the determinants with which it reacts are the outer amino acids in the molecule, which are probably subject to more variation with the species than those round the active centre.

Although the benzylamine/putrescine oxidases fail to show serological cross-reaction with the antibody to *C. boidinii* methylamine oxidase, they do show an inhibitor-sensitivity remarkably similar to that of the methylamine oxidases (the present work and Haywood & Large, 1981), and thus probably have similar active centres. Nonetheless, within this group of enzymes significant differences in substrate-specificity and other properties are apparent among the three enzymes that have been studied in this and a previous paper (Haywood & Large, 1981). In particular, the subunit molecular weight of the enzyme from *P. pastoris* is 50% larger than the subunit molecular weight of all the other enzymes (Table 4).

The immunological distinctions documented here contrast with the observations with yeast alcohol oxidases. Although the alcohol oxidases that have been purified from yeasts all show similar properties (Couderc & Baratti, 1980), three groups have been distinguished on the basis of electrophoretic mobility (Lee & Komagata, 1980; Komagata, 1980). Nevertheless the enzymes from *H. polymorpha* (Bringer *et al.*, 1979) and *P. pastoris* (Couderc & Baratti, 1980) both cross-reacted with antibody to purified alcohol oxidase from *C. boidinii*, suggesting a fairly close relationship. However, the number of yeast species possessing alcohol oxidase and able to grow on methanol as carbon source is relatively small [27 are mentioned by Komagata (1980) out of only four genera, *Candida*, *Torulopsis*, *Pichia* and *Hansenula*]. *Candida* and *Torulopsis* are now believed to be the same genus (Yarrow & Meyer, 1978), and according to Komagata (1980) the genera *Hansenula* and *Pichia* are closely related to one another. Thus in such a small group of yeasts it seems likely that a much smaller variation in properties might be expected in comparison with the much larger number of species (261 from 36 genera) that are able to use methylamine as nitrogen source (Van Dijken & Bos, 1981), which presumably all contain methylamine oxidase activity [although this has only been proved for some 18 species from ten genera (Yamada *et al.*, 1966; Green *et al.*, 1982)].

It seems likely that over the spectrum of the yeasts as a whole further differences in substrate-specificity will emerge, but that a basal pattern of two amine oxidases of related but significantly differing specificity will be found in most of the yeasts that can use primary amines as a nitrogen source for growth. The situation with growth on secondary amines is more complex. We have evidence that the oxidation of dimethylamine and trimethylamine is sensitive to CO (J. Green, unpublished work), and that in *C. boidinii* spermine and spermidine (when supplied as nitrogen sources) are oxidized by a different type of amine oxidase not sensitive to semicarbazide (G. W. Haywood, unpublished work).

The possibility of using benzylamine/putrescine oxidase from *P. pastoris* for the determination of spermine and spermidine was considered. Since its stoicheiometry for the oxidation of these compounds is identical, it would appear to offer advantages over plasma amine oxidase (Bachrach & Reches, 1966) (which oxidizes only the N^1-amino group of spermidine; Tabor *et al.*, 1964) because the aldehyde groups formed would give a direct measure of the total polyamines without the need to determine spermidine separately. It would also permit the simultaneous determination of putrescine, which is not possible with enzymic methods based solely on fungal polyamine oxidase (Isobe *et al.*, 1980). Unfortunately, the *P. pastoris* enzyme also oxidized lysine, which is a likely constituent of most systems containing polyamines, so that it cannot be applied to unfractionated biological mixtures. Moreover, the coupled peroxidase–ABTS system used in the present work, although very sensitive (it can detect 2 nmol of spermine or spermidine in a 3 ml reaction mixture), is interfered with by compounds present in urine, plasma or tissue extracts. This does not of course exclude the use of *P. pastoris* benzylamine/putrescine oxidase in place of plasma amine oxidase in the method of Bachrach & Reches (1966), nor does it prevent the use of the peroxidase–ABTS method in systems where interfering compounds are known to be absent. We have used it to measure the oxidation products of the polyamine oxidase of spermidine-grown *C. boidinii* after separation by ion-exchange (G. W. Haywood & P. J. Large, unpublished work).

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