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ON THE HETEROGENEITY OF THE TYROSINASE OF BROAD BEAN (*VICIA FABA* L.)

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Abstract—Multiple forms of the tyrosinase of *Vicia faba* have been shown to exist and a method for their partial separation is described. It has not been possible to distinguish the heterogeneity by determinations of their copper content, substrate specificity, degree of latency, or sedimentation. Evidence is presented which indicates that all the forms of tyrosinase contain one atom of copper per molecule of enzyme. Unlike manoxal, urea when used as activating agent appears to enhance the ability of the enzymes to oxidize monophenols.

INTRODUCTION

RECENT studies on tyrosinase have shown that the enzymes isolated from *Psalliota campestris*[1] or *P. bispora*[2] are quite distinct from those isolated from *Neurospora crassa*[3] and from the melanoma of mouse.[4] Most work has centred on the tyrosinase of *Psalliota* sp. and leads to the conclusion that four or five forms of the enzyme[2,5] are present in the tissue extracts. Two of those isolated from *P. bispora* have similar molecular weights, amino acid composition and each contains four atoms of copper per enzyme molecule.[2] These various forms of the enzyme can be differentiated by their relative ability to oxidize mono- and *ortho*-dihydric phenols. Several types of tyrosinase have also been found in *N. crassa* but these cannot be distinguished by kinetic analysis. Fox and Burnett[6] report that these forms can be prepared from a homocaryon, but Horowitz *et al.*[7] conclude that such a culture produces mostly one type. Examination of the tyrosinases isolated from cultures produced by various homocaryons shows that four types may be distinguished by their stability to heat and their electrophoretic mobility. Two of these forms, termed thermostable and thermolabile, have been extensively examined[3] and shown to have similar amino acid composition and molecular weight distribution. Both forms aggregate in solution, but in each case the major component in dilute solution (0·4% w/v) has a molecular weight of 33,000 ± 2000 and contains one molecule of copper per enzyme molecule. These two forms appear to have identical substrate specificities.[8] Although the data available for the enzyme isolated from mouse melanoma is more scanty the heterogeneity that is observed[4] resembles that described for *N. crassa*.

Since the above investigations were all carried out with lower plants or animals, it seemed desirable to examine the heterogeneity of tyrosinase in higher plants. It was decided to

[1] D. KERTESZ and R. ZITO, In *The Oxygenases* (Edited by O. HAYAISHI), Academic Press, New York (1962).
[2] S. BOUCHILLOUX, P. MCMAHILL and H. S. MASON, *J. Biol. Chem.* 238, 1699 (1963).
[3] M. FLING, N. H. HOROWITZ and S. F. HEINEMANN, *J. Biol. Chem.* 238, 2945 (1963).
[4] F. C. BROWN and D. N. WARD, *J. Biol. Chem.* 233, 77 (1958); *Proc. Soc. Exp. Biol. Med.* 100, 701 (1959).
[5] J. L. SMITH and R. L. KRUEGER, *J. Biol. Chem.* 237, 1121 (1962).
[6] A. S. FOX and J. B. BURNETT, *Biochem. Biophys. Acta* 61, 108 (1962).
[7] N. H. HOROWITZ, M. FLING, H. MACLEOD and N. SUESKA, *Genetics* 46, 1015 (1961).
[8] A. S. SUSSMAN, *Arch. Biochem. Biophys.* 96, 407 (1961).

examine the enzyme from the broad bean since the tyrosinase here is in a "latent" form[9,10] and its activity is greatly enhanced by treatment with certain denaturing agents.[11] Thus it is possible to prepare extracts which are mainly uncontaminated with oxidized phenols. A second reason for using broad bean is the possibility that it could throw light on whether *o*-hydroxylase activity is enzymic or not.

Isolation of the Enzyme

Unless stringent precautions are taken, extraction of tyrosinase from any plant tissue is accompanied by the oxidation of substrates to yield highly coloured polymeric quinones which react with proteins and are not easily removed: only a low yield of enzyme is thus obtained. A particular advantage of using the broad bean is that crude isolates show little tyrosinase activity; nevertheless since L-β-(3,4-dihydroxyphenyl-alanine) (DOPA) occurs in high concentration in the leaves (up to 9·4 g/kg fresh leaf tissue[9]) such isolates usually darken appreciably after 24 hr storage at +5°. In order to prevent this it was found necessary either to prepare an acetone powder and wash it with 80% ammonium sulphate solution to remove DOPA and any brown-coloured material before extraction of the enzyme, or to extract in the presence of ascorbate at pH 7·5[12], rapidly dialyse, precipitate with ammonium sulphate, centrifuge and lyophilize the water-soluble portion of the pellet. The enzyme was best extracted from either preparation with 0·1 M sodium borate buffer (pH 10·0).[13]

Demonstration of Heterogeneity in the Broad Bean Tyrosinase

When extracts of leaves, prepared as described above, are subjected to zone electrophoresis on starch gel and the gel treated with L-β-(3,4-dihydroxyphenyl)-alanine, at least four distinct sites of melanin production are identified (Fig. 1). Such heterogeneity has been observed in all the extracts which have been examined, whether made directly from fresh tissue or from acetone or lyophilized powders, and when electrophoresis has been conducted at any pH value between 4·8 and 8·0. At pH 5·0 the α fraction (see below) migrates towards the cathode and the β fraction towards the anode. These notations will be used in reference to fractions obtained in the course of enzyme purification.

Preparation of the Multiple Forms

It has not proved possible to prepare components which are absolutely homogeneous when examined by starch gel electrophoresis whether fractionation is carried out on TEAE, hydroxyl-apatite, carboxymethylcellulose, celite or zone electrophoresis on cellulose or by any combination of these methods. The method which was found to give the greatest purification utilized fractionation with ammonium sulphate followed by chromatography on triethylaminoethyl cellulose (TEAE cellulose) and hydroxyl-apatite. The combination of chromatography on these two adsorbents is desirable since the multiple forms which are more strongly adsorbed on TEAE-cellulose have the lowest affinity for hydroxyl-apatite.

(*a*) *Ammonium sulphate fractionation.* The lyophilized powder (see Experimental) was completely dissolved in 0·1 M borate, pH 10·0 (30 g/850 ml), and dialysed against two changes of 0·1 M borate, pH 8·0, and a final one of distilled water. A green precipitate which formed

[9] R. H. Kenten, *Biochem. J.* 67, 300 (1957).
[10] R. H. Kenten, *Biochem. J.* 68, 244 (1958).
[11] D. A. Ross, L. W. Mapson and T. Swain, *Nature* 207, 503 (1964).
[12] J. L. Bailey, *Biochem. J.* 79, 514 (1961).
[13] R. Macvicar and R. H. Burris, *Arch. Biochem.* 17, 31 (1948).

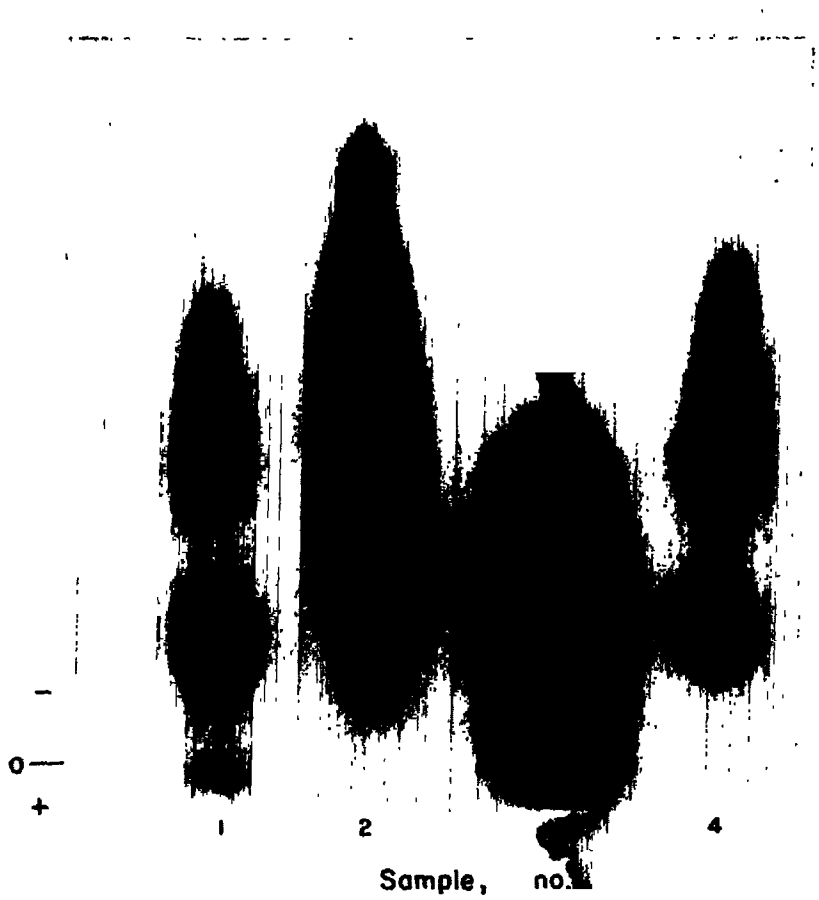

FIG. 1. SITES OF TYROSINASE ACTIVITY LOCATED ON STARCH GEL AFTER ELECTROPHORESIS OF A PREPARATION PURIFIED WITH AMMONIUM SULPHATE AND AGAIN AFTER ION EXCHANGE CHROMATOGRAPHY ON TEAE WHEN IT WAS FURTHER FRACTIONATED INTO α AND β COMPONENTS.

The analysis was performed overnight with a p.d. of 140 V at pH 6·4 using an 11% gel made up in a 0·005 M citric acid, 0·015 M Tris solution and employing 0·075 M maleate buffer, pH 6·4, in the electrode compartments. Samples applied (from left to right): (1) and (4) Preparation after ammonium sulphate purification; (2) component α; (3) component β. The origin is at O. At this pH all fractions migrate towards the cathode.

was discarded (volume of extract 870 ml). A 45–65% ammonium sulphate cut was taken, the precipitate completely dissolved in 0·025 M Tris-HCl, pH 8·0 (final volume, 190 ml), and the brown solution was dialysed against the same buffer (2 l.).

(*b*) *Ion exchange chromatography.* A 32 × 3·5 cm column prepared from TEAE-cellulose (40–100 mesh) was equilibrated with 0·025 M Tris-HCl (pH 8·0) and compressed under pressure (5 lb/in^2). The extract was applied to the column and washed in with 0·025 M Tris-HCl before 0·1 M Tris-HCl (pH 8·0) was added directly to the column. Extensive adsorption of brown material occurred at the top of the column; beneath it was a region of yellow material part of which was eluted by 0·1 M Tris-HCl yielding a fraction with tyrosinase activity, designated α (400 ml). The β fraction (340 ml) was eluted by a gradient generated by 0·5 M Tris-HCl (pH 8·0) passing into a mixing vessel containing 200 ml of 0·1 M buffer. Both fractions appeared to be heavily contaminated with nucleic acid since their u.v. spectra showed λ_{max} at 265 mμ with a shoulder at 280 mμ.

Both fractions were concentrated by precipitation with ammonium sulphate up to 65 per cent saturation. Nucleic acid-like material with maximum absorptivity at 265 mμ remained in the supernatant. The precipitates were dissolved in 0·01 M sodium phosphate buffer, pH 6·8, and dialysed against the same buffer (30 ml).

(*c*) *Adsorption chromatography on hydroxyl-apatite.* (i) *Fractionation of* α. The α fraction was introduced into a 16 cm × 2 cm column of hydroxyl-apatite which had been compressed under 5 lb/in^2 and equilibrated with 0·01 M sodium phosphate, pH 6·8. Much protein and some enzyme (designated α_1) was eluted by 0·05 M buffer before further components were eluted with a linear gradient developed with 0·1 M buffer (fraction α_2), and by direct elution with 0·2 M buffer, (α_3). Spectral examination of the three fractions showed that α_1 was still partly contaminated with nucleic acids while α_2 and α_3 gave broad maxima at 275 mμ.

(ii) *Fractionation of* β. Using a similar column, four cuts were taken from the TEAE-cellulose β-fraction. A little tyrosinase activity was found in the 0·01 M phosphate washings (β_0) but the majority of activity was recovered in two fractions (β_1 and β_2) eluted by stepwise gradients with 0·05 M and 0·1 M buffers; a small residual fraction (β_3) was eluted with 0·2 M buffer. All these fractions had more colour than the corresponding α fractions but their spectra showed one peak typical of proteins (λ_{max} at 274–276 mμ).

All seven fractions were concentrated by dialysis against powdered sucrose. The changes in specific activity during this three-stage fractionation are given in Table 1. It can be seen that the α_2 fraction has been purified three hundred and fifty times when compared to the extract made from the lyophilized powder—the overall purification when compared with a homogenate made directly from unfrozen leaves being over two thousandfold.

(*d*) *Fractionation using DEAE-cellulose and hydroxyl-apatite.* A disadvantage of the method described above is the low recovery (commonly 30–40 per cent) obtained from the TEAE-cellulose step. A change in the nature of the buffer does not improve the yield and no reason for the loss can be advanced. It is appropriate to note that low recoveries have been reported by other investigators.[4, 14, 15] Bailey,[12] however, has described a purification procedure involving fractionation on diethylaminoethylcellulose (DEAE-cellulose) where yields of 80–90 per cent were obtained when the column was operated at pH 7 and the tyrosinase was eluted by a pH and concentration gradient. Such a procedure fails to resolve the heterogeneity, but it has been used in the present work as an alternative to the TEAE-cellulose column described above. The tyrosinase activity obtained from such a fractionation, ($\alpha\beta$),

[14] E. Frieden and M. Ottesen, *Biochem. Biophys. Acta* **34**, 248 (1959).
[15] J. Porath and S. Hjerten, *Meth. Biochem. Anal.* **9**, 193 (1962).

Table 1. Purification of the broad bean tyrosinase α_2 and β_2

Step	Activity*	Protein (mg)	Specific activity	Activity yield (%)
Extract†	31,000	9,850	3·15	100
Ammonium sulphate	28,500	2,000	14	90
TEAE				
α	6,000	164	37	
β	2,900	270	11	
Total	8,900	434		29
Hydroxyl apatite				
α^1	230	3·3	70	
α_2	5,600	5·3	1,100	
α_3	560	0·9	620	
β_0	420	22	19	
β_1	1,080	30	36	
β_2	1,390	3·5	400	
β_3	60	0·8	70	
Total	9,340	65·8		30

* With homocatechol.
† 30 g of lyophilized powder.

Table 2. Purification of the tyrosinase fractions $\alpha\beta_1$ and $\alpha\beta_2$

Step	Volume (ml)	Activity* (units)	Protein (mg)	Specific activity (units/mg)	Activity yield (%)
Extract†	1,600	47,500	9,300	5·1	100
Ammonium sulphate	260	39,500	2,500	15	83
DEAE					
First column retained		20,110	560	36	
discarded		3,900	230	17	
Total		24,010	790		51
Second column		13,200	445	30	28
Hydroxyl-apatite					
$\alpha\beta_1$		2,580	25	104	
$\alpha\beta_2$		7,140	11·5	620	
Total (with $\alpha\beta_0$ and $\alpha\beta_3$)		10,740	36.5		23
Zone electrophoresis on cellulose					
$\alpha\beta_1$		1,900	9·8	195	
$\alpha\beta_2$		3,000	5·9	510	
Total		4,900	16·7		10

* With homocatechol.
† 60 g of lyophilized powder.

was then subjected to further fractionation on hydroxyl-apatite as described above giving fractions $\alpha\beta_0$, $\alpha\beta_1$, $\alpha\beta_2$ and $\alpha\beta_3$. Data in Table 2 shows that this mode of purification gives only half the specific activity for the $\alpha\beta_2$ compared with α_2 (Table 1), but for $\alpha\beta_1$ it exceeds that obtained for either α_1 or β_1. Attempts to purify further using zone electrophoresis on cellulose[15] gave poor recoveries.

Some Properties of the Purified Fractions

The properties reported here are mainly those of the α_2, $\alpha\beta_1$ and $\alpha\beta_2$ fractions whose purification is described above. The starch gel patterns of $\alpha\beta_1$ and $\alpha\beta_2$ were similar to β_2 and α_2 respectively.

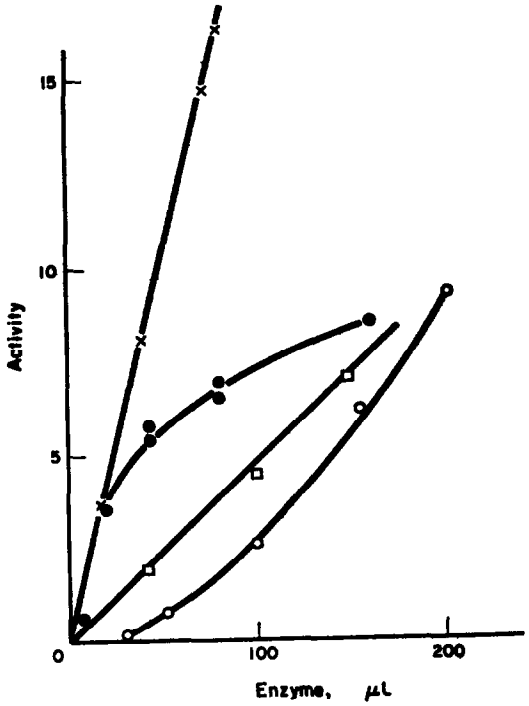

FIG. 2. THE RELATIONSHIP BETWEEN ACTIVITY AND ENZYME CONCENTRATION DETERMINED IN THE PRESENCE AND ABSENCE OF MANOXAL.

Activity with homocatechol with ×—× and without ●—● manoxal measured with the oxygen cell: activity with *p*-cresol with □—□ and without ○—○ manoxal measured manometrically.

(*a*) *Activity before activation.* Although many denaturing agents have been shown to enhance the low level of activity observed in crude enzyme extracts the purified forms possess a high degree of activity in the absence of such agents, and accordingly little additional activation can be achieved. Manoxal treatment of the α_1, $\alpha\beta_1$ and $\alpha\beta_2$ fractions only enhances the homocatecholase activity two-fold. However, the complication exists that, in contrast to assays made in the presence of activating agents, a linear response was not observed between enzyme concentration and activity when manoxal was omitted, see Fig. 2.

(*b*) *Homocatecholase: cresolase ratio.* Homocatechol and *p*-cresol are oxidized more readily by broad bean tyrosinase than any other *o*-dihydric or monohydric phenol and so these

two compounds were used to compare the ability of broad bean tyrosinase to dehydrogenate diphenols and hydroxylate monophenols. Values obtained for the ratio of these activities, measured both in the presence of manoxal or urea and in their absence, are given in Table 3. Whilst significantly different values were obtained depending on the agent used, the purified fractions α_2 and β_2 behave similarly and there is no great difference for any single fraction with manoxal.

Table 3. Activity and homocatecholase–cresolase ratios of the various purified forms

Enzyme fractions	Oxygen uptake μM/min/mg protein	Homocatecholase/cresolase ratio in the presence of: Manoxal	Urea (4 M)	No agent
α_2	500	22	4·7	6·3
β_2	170	20	7	
$\alpha\beta_1$	115	25		
$\alpha\beta_2$	376	15		

(*c*) *Absorption spectra.* All the purified fractions so far examined are pale yellow in colour and have only one absorbance maximum (λ_{max} = 275–276 mμ) with a small shoulder at 290 mμ (see Fig. 3). Thus the broad bean tyrosinases can be distinguished from the blue phenol

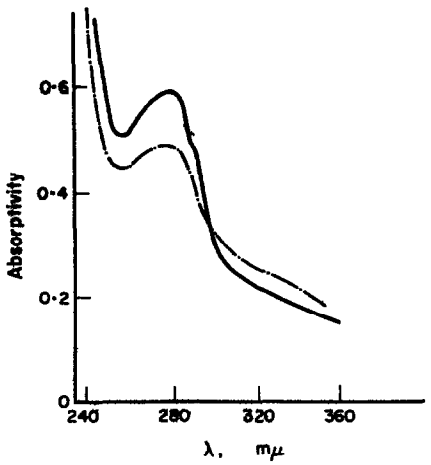

Fig. 3. The absorption spectra of fractions α_2 (——) and β_2 (–.–) in 5 mM phosphate, pH 8·0. The protein concentrations of α_2 was 0·71 mg/ml; that of the less pure β_2 fraction was not determined.

oxidase of tea [16] and the tyrosinases of *N. crassa* and *Psalliota* species which show maxima at 280 mμ with very distinctive shoulders at 290 mμ,[2,3] but they are in this respect similar to the enzyme isolated from potato.[17] The absorptivity of 1% solutions of α_2, $\alpha\beta_2$ and $\alpha\beta_1$ at 275 mμ

[16] D. S. Bendall and R. P. F. Gregory, In *Enzyme Chemistry of Phenolic Compounds* (Edited by J. Pridham), Pergamon Press, Oxford (1963).
[17] F. Kubowitz, *Biochem. Zt.* **299**, 32 (1938).

and pH 6–8 was 8·2, 12·0 and 14·3 per cm respectively. There is insufficient data available to correlate these differences with heterogeneity, and the distinct possibility exists that it is related only to the purity of the specimens for part of the colour may be due to adsorbed impurities. Thus, α_2 fraction which had the highest specific activity of these components also possesses the lowest absorptivity. The absorptivity values may be compared with those found for the tyrosinases from mouse melanoma, *Psalliota* sp. and *N. crassa*, namely 0·88,[4] 24·5–27·5[1,2] and 19·0 respectively.

(*d*) *Ultracentrifuge studies.* Three preparations were studied by the sedimentation velocity method and in all cases only one sedimenting peak was observed. The sedimentation coefficients corrected to 20° for fractions $\alpha\beta_1$ and $\alpha\beta_2$, and for a preparation purified one hundredfold and containing all the tyrosinase forms, were found to be 3·8, 4·1 and 4·3* respectively. The determinations were made in an artificial boundary cell using a Spinco Model E centrifuge at pH 6, at a protein concentration approximating in all cases to 10 mg/ml and a speed of 52,640 rev/min. Such data indicate that the multiple forms are of similar molecular weight distributions and are not polymers of one another.

(*e*) *Determination of constituents.* Analyses for protein, carbohydrate and copper were performed using the fractions $\alpha\beta_1$ and $\alpha\beta_2$ and a third preparation (G) containing all components and having an overall specific activity of 190 μmoles O_2/min/mg protein. The latter specimen was made from an acetone powder, purified by pH and gradient elution from TEAE and by two fractionations on hydroxyl-apatite from which it was eluted by direct application of 0·1 M sodium phosphate buffer.

TABLE 4. CONSTITUENTS OF THE PURIFIED FORMS

Enzyme fraction	Dry weight (mg)	Protein* (mg)	Carbohydrate* (as % of protein)	Copper† (as % of protein)
$\alpha\beta_1$	3·5	3·6	3·8	0·14
$\alpha\beta_2$	6·1	5·9	—	0·15
G		2·5	3·7	0·14

* Average of three determinations.
† One determination only.

As Table 4 shows, a similar quantity of copper (0·14–0·15 per cent) was found in all preparations—an amount which has been reported previously for some tyrosinase preparations from other sources, but which nevertheless is lower than that more generally found (0·19–0·21 per cent). An attempt to determine the amino acid composition of fraction $\alpha\beta_1$ using ion-exchange chromatography and thereby obtain its minimum molecular weight was marred by the low (66 per cent) recovery of the nitrogen from the column. A large amount of ammonia was present in the hydrolysate which may indicate that some decomposition had occurred. Data for the amino acid composition of the tyrosinases of *N. crassa* and *P. bispora* show that in both cases only small amounts of histidine and methionine are found and cystine is virtually absent; we were unable to detect methionine and cystine in our analyses and histidine formed less than 1·5 per cent of the amino acids recovered.

* This value was incorrectly reported in a previous communication[11] as 2·04.

Attempted Conversion of the Multiple Forms

Several examples are known of the interconversion of one form of enzyme into another. A notable instance concerns the interconversion by freezing and thawing saline solutions of the five forms of the lactic acid dehydrogenase of mouse,[18] and another involving the conversion of two forms of the tyrosinase of *N. crassa* into a third form has been described by Fox and Burnett.[6] Freezing portions of the $\alpha\beta_1$ fraction in 6 M urea, pH 4·7, 2 M sodium chloride, pH 5, and 5 M acetate, pH 5, and examining the starch gel pattern obtained after thawing and again after dialysis showed that a component resembling $\alpha\beta_2$ was not produced by any treatment. Similarly when an α fraction was activated in urea at pH 4·7 starch gel analysis showed that a component of higher mobility was produced but on removal of the urea the pattern characteristic of an α fraction was reproduced.

DISCUSSION

It is convenient to classify the copper oxidases which oxidize *ortho*-dihydric phenols but not ascorbic acid into two groups distinguished by the added ability of one group to catalyse the *ortho*-hydroxylation of monophenols. The substrate specificity of the broad bean complex indicates that it is a member of this group commonly termed 'tyrosinases'. This evidence is reinforced by the pale yellow colour of the purified fractions which is typical of the mushroom and *Neurospora* enzymes and is in contrast to the blue colour associated with the laccases and phenol oxidase of tea which are unable to catalyse the hydroxylation reaction.[15]

Within the tyrosinase group physico-chemical data are available for enzymes from the three sources, mushroom, mouse and *N. crassa*, and on comparison with data reported in this paper it seems that the broad bean enzymes more closely resemble the enzymes of mouse and *Neurospora*. For example the sedimentation data indicate that the various forms of broad bean tyrosinase are similar in size to each other, to the major component of mouse melanoma ($S = 3{\cdot}4$) and to the two forms of *N. crassa* which have been investigated (S_{20} is 4·3 S when determined by the sedimentation velocity method) and different from the forms isolated from *P. bispora* ($S_{20} = 6{\cdot}4$ S). Such variation in size is in contrast to the consistency of the copper composition and, as mentioned earlier, it is suggested that the tyrosinases of mushroom and *N. crassa* contain four and one atoms of copper per molecule respectively. Providing that the shapes of the broad bean enzymes are not vastly different from those in *N. crassa* it is likely that these also contain one atom of copper per molecule. This is reinforced by a consideration of the specific activities reported for the broad bean and *Neurospora* enzymes. It has already been noted that the oxygen consumption of the α_2 fraction when oxidizing homocatechol at 25° was 500 μmoles/min/mg protein (Table 3). *Neurospora* tyrosinase oxidizes catechol and DOPA at similar rates, the best preparation of Fling *et al.*[3] oxidized 536 μmoles of DOPA per minute at 30° (judged by the formation of dopachrome) and they showed that under these assay conditions one molecule of oxygen was consumed for each molecule of DOPA oxidized. The corresponding figure obtained manometrically, for a high catecholase mushroom enzyme oxidizing its best substrate (catechol) in the presence of gelatine at 25° is surprisingly low, namely 754 μmoles/min/mg protein.[2]

The nature of the heterogeneity of broad bean tyrosinase is also similar to *Neurospora* and unlike mushroom in that whilst various forms can be distinguished on properties primarily due to the tertiary structure of the molecules, this difference is not reflected kinetically with any substrate. Thus at the moment the multiple forms of broad bean tyrosinase are

[18] C. L. MARKERT, *Science* **140**, 1329 (1963).

recognized mainly by their charge distribution and not by their size, copper content or activity. An obvious explanation of this heterogeneity is that it is an artefact of preparation. Bendall and Gregory[16] have isolated both blue and yellow fractions from tea leaf and both display phenol oxidase activity. They suggest that the native enzyme is blue, and that when the leaves are disrupted yellow tanned enzymes are produced by the reaction between proteins and the quinones formed by the phenol oxidase. Since the purification described above leads to the separation of fractions with varying specific activities it could be argued that a similar explanation could be applied and that the native enzyme is the fraction (α_2) with the highest specific activity. However, stringent precautions have been taken during isolation to minimize quinone formation and it is thought unlikely that this hypothesis provides a satisfactory explanation. That the forms are produced during the purification process is also thought to be unlikely since the starch gel patterns are consistently as expected and recycling the enzyme fractions down fresh columns does not change the elution characteristics. It is possible that the various forms may be distinguished by the number of glutamic and aspartic acids which occur as asparagine and glutamine moieties but this aspect has not been investigated.

One other interesting feature which emerges from this study is that the relative ability of a tyrosinase to oxidize monophenols and *ortho*-diphenols is influenced by denaturing agents. Thus the homocatecholase and cresolase activities determined in the absence of "activating" agents are stimulated when urea and manoxal are added, but the value of the ratio of the two activities also alters suggesting that the two activities behave differently. It can also be shown that the activity of potato tyrosinase, which is not enhanced by treatment with anionic detergents, is also modified in the presence of urea. Thus when the homocatechol-cresol ratio of a crude potato extract is determined in 8 M urea it is about half the value obtained when urea is omitted. This distinctive effect of urea contrasts with the failure of competitive inhibitors and treatment causing reaction inactivation to distinguish the two activities in mushroom tyrosinase.[19]

EXPERIMENTAL

Materials. Homocatechol and *p*-cresol were commercial samples supplied by Lights and B.D.H. respectively, and both were purified before use to constant melting point (105° and 36–37° respectively). The column adsorbents TEAE-cellulose and hydroxyl-apatite were obtained from Serva. Other chemicals used were of the finest grade available and all aqueous solutions were made with once distilled column-deionized water.

Preparation of Crude Enzyme

Acetone powders were prepared by freezing the leaves at −20°, mincing at −3° and adding to acetone (20 ml/g) at −20°. The acetone insoluble material was recovered as a free flowing powder after filtration and drying at room temperature. Immediately before extraction of the enzyme the acetone powder was washed with 80% $(NH_4)_2SO_4$ solution. Lyophilized powders were prepared from the frozen minced leaf by extraction with 0·005 M sodium phosphate buffer, pH 7·5, containing ascorbic acid (0·028 M). After dialysis against ice-water the extract was saturated with ammonium sulphate, centrifuged, and the resultant pellet dissolved in water and freeze dried. Crude extracts were obtained from acetone and lyophilized powders by extraction with 0·1 M sodium borate (pH 10·0).

[19] D. C. Gregg and J. M. Nelson, *J. Am. Chem. Soc.* **62**, 2500 (1940).

Assays of Enzyme Activity.

Routine assays were conducted with an oxygen electrode of the Clark type[20] at 25°. To 3·0 ml of enzyme in 0·1 M phosphate–0·05 M citrate buffer, pH 5·0, was added 0·1 ml of a 0·01 M solution of manoxal (bis-(2-ethylhexyl) sodium sulphosuccinate), followed 1 min later by 0·4 ml of either homocatechol or *p*-cresol (10 mg/ml). The initial oxygen concentration was equivalent to pO_2 (air) of 0·21. The rate of decrease in the current (which is directly proportional to the rate of O_2 uptake) was followed on a 0–1 mV recorder. Only the initially linear portion was used in the determination of enzyme activity. One unit of activity is defined as that amount which produces an oxygen uptake equivalent to a drop in potential of 1 mV/min. In determinations made by manometric methods the enzyme sample and manoxal were added from the sidearm to buffer and substrate (total vol 3·0 ml). With homocatechol and *p*-cresol the presence of alkali in the centre well was found to be unnecessary. When manoxal was replaced with urea, the latter agent was employed in final concentrations of 5 M and this necessitated changes in the technique. For example using the oxygen electrode the enzyme solution was incubated with urea, buffered at pH 4·7 with 0·1 M phosphate 0·05 M citrate for 15 min before the substrate was introduced. In the manometric method the enzyme was added from the sidearm to a mixture of buffer, urea and substrate again at pH 4·7. When the activating agent was omitted determinations were made with the manometric method at pH 4·5 in 0·1 M acetate buffer. When *p*-cresol was used as a substrate the reaction proceeded at a linear rate after an induction phase. In determining cresolase activity, this induction phase has been disregarded.

Estimation of Dry Weight, Protein, Carbohydrate and Copper

The dry weight of certain preparations after dialysis to remove salts was estimated by freezing the sample and drying it to constant weight *in vacuo* over conc. H_2SO_4. The protein content was determined according to the method of Lowry *et al.*[21] using bovine serum albumin as standard. Carbohydrate material was measured with anthrone as described by Yemm and Willis,[22] employing lactose as a standard. The method of Stark and Dawson[23] was adopted for the determination of copper.

Starch Gel Electrophoresis

Most determinations were made at pH 8 according to the procedure of Smithies[24] as modified by Poulik,[25] except that several other minor changes were made. Thus electrophoresis was conducted in the horizontal plane at +5° using a 11% hydrolysed starch gel made in a solution 0·11 M with respect to Tris and 0·007 M with respect to critic acid. When other pH values were used a discontinuous buffer system was retained and thus at pH 5 the gel was made in a solution containing 0·005 M ethylenediamine tetra-acetic acid neutralized with Tris to pH 5·1, and 0·3 M acetate was present in the electrode compartments.

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