The Purification and Properties of a Ribonucleoenzyme, *o*-Diphenol Oxidase, from Potatoes

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(Received 6 January 1970)

1. *o*-Diphenol oxidase was isolated from potato tubers by a new approach that avoids the browning due to autoxidation. 2. There are at least three forms of the enzyme, of different molecular weights. The major form, of highest molecular weight, was separated from the others in good yield and with high specific activity by gel filtration through Bio-Gel P-300. 3. The major form is homogeneous by disc electrophoresis but regenerates small amounts of the species of lower molecular weight, as shown by rechromatography on Bio-Gel P-300. 4. There is an equal amount of RNA and protein by weight in the fully active enzyme. The RNA cannot be removed without loss of activity, and is not attacked by ribonuclease. 5. The pH optimum of the enzyme is at pH 5.0 when assayed with 4-methylcatechol as substrate. It is ten times more active with this substrate than with chlorogenic acid or catechol. The enzyme is fully active in 4M-urea. 6. A minimal molecular weight of 36000 is indicated by copper content and amino acid analysis of the protein component of the enzyme. 7. The protein contains five half-cystinyl residues per 36000 daltons, a value similar to that found in *o*-diphenol oxidase from mushrooms. It also contains tyrosine residues although, when pure, it does not turn brown by autoxidation.

It has often been assumed that *o*-diphenol oxidases do not contain cysteinyl or cystinyl residues. For example Kertesz & Zito (1962), although not excluding the possibility of some disulphide groups in the enzymes, could find no evidence for thiol groups, and concluded that if half-cystinyl residues were present they could not be numerous and had no importance for the enzyme activity. Fling, Horowitz & Heinmann (1963) concluded from amino acid analyses of the performic acid-oxidized enzyme from *Neurospora* that there was no half-cystine present.

Recent findings, however, that the mushroom enzyme does contain half-cystine (Kertesz, 1966; Jolley, Robb & Mason, 1969*a*) have reopened the question. The present work started as a re-investigation of the methods of isolating the potato enzyme, with a view to investigating its amino acid composition.

One of the main problems encountered in the isolation of *o*-diphenol oxidases is the fact that they catalyse the oxidation of proteins, possibly including themselves, giving rise to the phenomenon of enzymic browning or tanning. Naturally occurring phenolic substrates, such as chlorogenic acid in the potato, can mediate this process by becoming oxidized to *o*-quinones, which then react with

amino acid residues, such as tyrosyl, lysyl, or cysteinyl residues, in the protein molecules. Alternatively, exposed tyrosyl residues in proteins may be directly oxidized via dopa (3,4-dihydroxyphenyl-alanine) to dopaquinone residues, which may then react with the side chains already mentioned (Sizer, 1953). Such processes could lead, not only to the possible loss of thiol or other groups in the *o*-diphenol oxidase molecule itself, but to induced heterogeneity and to loss of enzyme activity.

For these reasons it is important to take every precaution to avoid enzymic browning during isolation. The preparative procedure has been designed with this in mind. It has been found that the major concentration of phenols is found just beneath the skin of the potato (Johnson & Schaal, 1952), so even though the peelings contain a greater concentration of the enzyme than the peeled potato (Kubowitz, 1937) they were discarded. Low-molecular-weight phenols were removed as rapidly as possible after homogenization of the potato and the enzyme was maintained in an inhibited state during purification. The readily removed competitive inhibitor, benzoic acid (Kuttner & Wagreich, 1953) was employed for this purpose.

The development of a new isolation procedure has led to the study of some properties of the pure

enzyme, including its copper content and amino acid composition, and to the unexpected finding that a large amount of RNA is associated with the enzyme and may be necessary for its activity.

MATERIALS

Potatoes. Attested virus-free potatoes, King Edward variety, were obtained from the Department of Agriculture and Fisheries, East Craigs, Edinburgh, U.K.

Reagents. All reagents were AnalaR or Biochemical grade (BDH Chemicals Ltd., Poole, Dorset, U.K.) except the following: bovine plasma albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.); Coomassie Brilliant Blue (Imperial Chemical Industries Ltd., Dyestuffs Division, Blackley, Manchester, U.K.); Hyflo Supercel (Johns-Manville, Lampoc, Calif., U.S.A.); salmon sperm DNA (Calbiochem, Los Angeles, Calif., U.S.A.); ribonuclease (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany); Bio-Gel P-300 (Bio-Rad Laboratories, Richmond, Calif., U.S.A.). The following were Laboratory grade reagents (BDH Chemicals Ltd.): K_2HPO_4 ; NNN' -tetramethyl-1,2-diaminoethane (TEMED); 4-methylcatechol. The last-named was recrystallized from toluene before use.

Glass-distilled water was used throughout.

METHODS

Enzyme assay. A modification of the indirect spectrophotometric assay of Sisler & Evans (1958) was used. The decrease in extinction was followed at 265 nm and 25°C with a Beckman DB spectrophotometer and a Sargent Recorder. The standard reaction mixture (3.0 ml) contained 80.7 mm-4-methylcatechol, 32 μ M-ascorbic acid, 0.32 mm-EDTA and 32 mm-potassium phosphate buffer, pH 6.8. The blank contained all the above except ascorbic acid. All solutions were equilibrated with atmospheric oxygen at 25°C before use. Reaction was started by adding 0.10 ml of the enzyme solution.

Enzyme unit (U). This is the international unit defined as the amount of enzyme that catalyses the conversion of 1 μ mol of 4-methylcatechol/min. This corresponds to a decrease of 5.25 extinction units/min at 265 nm in this assay, since the molar extinction coefficients of ascorbic acid and dehydroascorbic acid are 1.65×10^4 and 2.3×10^2 respectively, under these conditions.

Correction for inhibition by benzoic acid. Where benzoic acid was present in the enzyme solution assayed, the

observed values of activity were corrected. Fig. 1 shows the standard curve correlating the percentage inhibition with the concentration of benzoic acid present in the enzyme solution assayed.

Protein concentration. This was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Isolation of the enzyme. Unless otherwise stated, all the buffers used in the purification procedure were made up in water containing benzoic acid (1.8 g/l). The compositions of buffers used are given in Table 1.

Stage 1. Peeled potato slices (2 kg) were homogenized in a Waring Blendor with 2 litres of buffer I (see Table 1). The suspension was left for 1 h with occasional stirring and passed through a layer of surgical gauze to remove large cell debris. To every litre of suspension was added 90 g of Hyflo Supercel. The slurry was then filtered through a 6.5 cm-diameter stainless-steel mesh (13 μ m equivalent pore size; Sankey Green Wire Weaving Co. Ltd., Thelwall, Warrington, U.K.) under a nitrogen pressure of 172 kN/m² (25 lb/in²) in a heavy-walled glass column. The clear filtrate had no activity and was discarded. The packed material in the filter was washed with 50 ml of buffer II, after which activity was eluted from the column with buffer III.

Stage 2. Fractions (10 ml) were collected and all those containing activity were pooled. The first few active fractions always contained a very fine precipitate. Ammonium sulphate was added to the pooled active fractions (25 g/100 ml) and the active precipitate was collected by centrifugation at 23 000 g for 40 min at 0–5°C.

Stage 3. At this point the precipitate was usually divided into portions (8–10 g) to avoid overloading the Bio-Gel P-300 column used in the final stage. A portion was transferred into a dialysis tube with the minimum volume of buffer III and dialysed against the same buffer. Any precipitate present was removed by centrifugation at 108 000 g for 20 min at 0–5°C.

Stage 4. An equal volume of 2% (w/v) protamine sulphate in buffer III was added. After standing for 4–5 h with occasional stirring, the suspension was centrifuged as before.

Stage 5. The supernatant solution was concentrated to a final vol. of 5 ml in a Diaflo ultrafiltration cell and recentrifuged at 200 000 g for 40 min at 0–5°C. The concentrated solution was applied to a column of Bio-Gel P-300 (2.5 cm $\times$ 40 cm) equilibrated with buffer III, and eluted with the same buffer. Fractions (3 ml) were collected and assayed for activity.

Stage 6. The first active peak contains the purified enzyme (see the Results section). The course of a typical

Table 1. *Compositions of buffers used in the purification procedure*

All these buffers contained benzoic acid (1.8 g/l of water).

	pH	$(NH_4)_2SO_4$ (g/l of water)	K_2HPO_4 (g/l of water)	Sodium acetate tri-hydrate (g/l of water)
Buffer I	7.0*	700	26	—
Buffer II	7.0*	370	17.5	—
Buffer III	5.6	—	—	20.4

* pH was adjusted with 35% NH_3 solution.

Table 2. *Typical preparation of o-diphenol oxidase, starting with 2 kg of peeled potatoes*

Purification stage	Volume (ml)	Activity (U/ml)	Total activity (U)	Protein (mg/ml)	Specific activity (U/mg)	Recovery (%)
1. Starting material	—	—	2220*	—	0.42*	100
2. Pooled active fractions from filter	920	1.96	1810	2.0	0.98	81
3. Supernatant before protamine sulphate treatment	45	31.2	1410	38	0.82	63
4. Supernatant after protamine sulphate treatment	80	14.5	1160	20	0.73	52
5. Solution applied to Bio-Gel P-300	30†	38.1	1140	36	1.06	51
6. Pooled peak from Bio-Gel P-300	108	16.1	1740	0.185	87	78

* Extractable with 0.1 M-potassium phosphate buffer, pH 7.0, instead of buffer I.

† Applied in 5.0 ml portions in six successive experiments.

purification is shown in Table 2. About 20 mg of purified enzyme protein is obtained from 2 kg of starting material. The specific activity of this material is between 80 and 120 U/mg, and although stable in concentrated solutions it loses activity quickly in dilute solutions (see the Results section).

RNA concentration. This was determined by the orcinol reaction (Schneider, 1957) with yeast RNA as standard.

DNA concentration. This was measured by the method of Burton (1956) with salmon sperm DNA as standard.

Disc electrophoresis. A modification of the technique of Davis (1964) was adopted, in which the sample and spacer gels were omitted. The gels contained either 4% acrylamide and 0.08% bisacrylamide, a 4% by 2% gel (Hjertén, 1962), or 7.5% acrylamide and 0.0375% bisacrylamide, a 7.5% by 5% gel. In either case 0.075% ammonium persulphate and 0.05% TEMED were present and the gels were prepared in 0.05 M-tris-HCl buffers of the required pH. Before application of samples to the gels, ammonium persulphate was removed by passing a current of 5 mA/tube for 1 h with 0.5 M-tris-HCl buffer in the electrode compartments. Samples were layered directly on top of the gels and electrophoresis was carried out with 0.05 M-tris-glycine buffer in the electrode compartments. Bromophenol Blue was used as an indicator.

Active bands in the gel were detected by a staining procedure (Jolley & Mason, 1965). Freshly extruded gels were immersed in a solution of 4-methylcatechol (1.0 mM) and L-proline (1.0 mM) in 0.1 M-potassium phosphate buffer, pH 6.5. After 1–2 min a purple colour developed in the gel at the site of activity. Inactive protein bands were not stained.

Protein bands were located by staining with Coomassie Brilliant Blue [0.05% (w/v) in 10% (w/v) trichloroacetic acid] for 30–90 min (Chrambach, Reisfeld, Wyckoff & Zacari, 1967), followed by removal of unbound dye with several changes of 10% (w/v) trichloroacetic acid.

Digestion with ribonuclease. The assay method of Kunitz (1946) was employed to check the activity of the ribonuclease used. Digestion of yeast RNA by the enzyme (about 0.01 mg/ml) was followed spectrophotometrically at 300 nm and 25°C in the presence of sodium acetate buffer. In these experiments the buffer pH was 5.6 instead of 5.0 as recommended by Kunitz, and benzoic acid was present. The activity of the ribonuclease was 30 Kunitz units/mg under these conditions.

Treatment of various solutions with ribonuclease was carried out under conditions essentially similar to those described above, except that higher ribonuclease concentrations were sometimes used.

Copper determination. The method of Stark & Dawson (1958) as adapted by Gregory & Bendall (1966) was used, with $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ as standard.

Dry-weight determinations of protein. These were carried out as described by Gregory & Bendall (1966).

Amino acid analysis. Before acid hydrolysis, RNA and copper were removed from the protein as follows: trichloroacetic acid solution [50% (w/v)] was added to the enzyme solution containing 1–2 mg of protein, to give a final concentration of 5% trichloroacetic acid. The resulting solution was left at 0–4°C overnight to complete the precipitation of protein. The precipitate was removed by centrifugation (2000g for 15 min) and suspended in 2 ml of 0.2 M-tris-HCl buffer, pH 8.5. The suspension was dialysed against 1.0 M-KCN for 5 h and then exhaustively against water before being freeze-dried. Hydrolysis was carried out as described by Moore & Stein (1963) and performic acid oxidation by the method of Moore (1963). Amino acid analysis was performed with an EEL 199 amino acid analyser, by the method of Atkin & Ferdinand (1969).

RESULTS

Preparative procedure. The presence of benzoic acid in the buffers during the isolation procedure and storage of the enzyme inhibits enzymic browning of the solutions. When such solutions are assayed by the routine method 62% inhibition is observed if the 0.1 ml of enzyme solution added to the cuvette is saturated with benzoic acid. If lower concentrations of benzoic acid are present, e.g. after dilution of the enzyme solution with water

Fig. 1. Inhibition of *o*-diphenol oxidase activity by benzoic acid. The benzoic acid concentration shown on the abscissa is the concentration in the enzyme solution assayed (0.1 ml) in the standard assay described in the text (3.1 ml total volume).

before assay, the inhibition decreases as shown in Fig. 1.

The presence of ammonium sulphate in buffer I ensures the immediate precipitation of the enzyme during homogenization of the potato. These two factors decrease browning to a very low value. Previous workers (Alberghina, 1964; Abukharma & Woolhouse, 1966) have used ascorbic acid in their solutions to prevent browning during the isolation of the enzyme, but this was found to be much less effective, probably owing to rapid exhaustion of the ascorbic acid. The ammonium sulphate precipitation and pressure filtration of the homogenate not only remove phenols present in the tissue as soon as possible, but also decrease the amount of centrifugation involved. Hyflo Supercel does not inactivate the enzyme. In spite of the precautions taken some darkening of the solution does take place, but most of the dark-coloured material remains insoluble after redissolving the ammonium sulphate precipitate at stage 3. About 20–30% of the activity is associated with the brown precipitate.

During the protamine sulphate treatment of the material at stage 4, 50% of the RNA present at the end of stage 3 is removed. The addition of more protamine sulphate, or streptomycin sulphate, fails to remove any more RNA.

Volumes of 5 ml or less were applied to the Bio-Gel P-300 column to obtain the maximum resolution from the column. The use of longer or wider columns is not recommended as it results either in very slow elution or in peak spreading. Activity was eluted from the column in three, or possibly more, peaks, the first and major one of which emerged at the void volume of the column and had the highest specific activity. The later active peaks were probably contaminated with inactive protein as shown in Fig. 2, which also shows that RNA is associated with the active peaks. Essentially the

Fig. 2. Typical elution pattern obtained in stage 5 of the preparation of *o*-diphenol oxidase from potatoes. A portion (5.0 ml) of the partially purified enzyme solution was applied to a Bio-Gel P-300 column (2.5 cm $\times$ 40 cm) and eluted with buffer III of Table 1. $\circ$, Activity; $\bullet$, protein; Δ , RNA.

same pattern of activity, protein and RNA was obtained from a sample that had been treated with ribonuclease before application to the column, suggesting that the RNA associated with the various peaks is actually bound to protein. Further evidence for this is given below under 'Absorption spectrum and nucleic acid content'.

Rechromatography on Bio-Gel P-300. Rechromatography of the purified enzyme gave three active peaks in the same positions as the first three shown in Fig. 2 with 95% recovery of activity. The same result was obtained in the absence of benzoic acid, or if 4M-urea was present in buffer III, or if buffer III was replaced by 0.1M-potassium phosphate buffer, pH 7.0. The enzyme is fully active in the presence of 4M-urea. The first of the three peaks was always the major one and had the highest specific activity. No inactive protein peaks were observed.

Stability of the enzyme. The enzyme is very stable up to stage 5 of the purification procedure and the purified enzyme loses only 1.75% of its activity per day for several days at concentrations greater than 1 mg/ml in either buffer III (Table 1) or potassium phosphate buffer, pH 7.0 (benzoic acid present in both cases), and at either 0°C or –15°C. In dilute solutions (0.15–0.20 mg/ml), however, it loses activity rapidly in either buffer at either temperature. For example in buffer III at 0°C, 35% of the activity has been lost after one day and a further 30% after a second day.

A problem arises in the final stage of the purification because the enzyme is eluted from the Bio-Gel P-300 column at concentrations in the low, unstable,

Fig. 3. Spectrum of *o*-diphenol oxidase in 0.15 M-sodium acetate buffer, pH 5.6.

range. As soon as the enzyme has been eluted the fractions must be pooled and concentrated at 0°C. Once inactivation has occurred in dilute solutions, concentration does not restore activity. The most convenient way of concentration is by ultra-filtration.

Absorption spectrum and nucleic acid content. The E_{260}/E_{280} ratio for the purified enzyme is about 1.1 (Fig. 3). There is no absorption in the visible region, indicating that the protein has not become tanned during isolation. It is significant that although crude preparations of the enzyme rapidly turn brown on standing in the absence of benzoic acid, the purified enzyme does not. This does not mean that the enzyme cannot oxidize its own tyrosyl residues, but only that the subsequent coupling reactions leading to colour cannot occur. The pure enzyme is normally stored in the presence of benzoic acid.

The enzyme contains equal amounts by weight of RNA and protein, but no DNA can be detected. The RNA cannot be removed from the protein by methods that preserve its enzymic activity, though precipitation of the protein with trichloroacetic acid leaves the RNA in solution. Attempts at digestion of the enzymically active protein-RNA complex with ribonuclease (up to 1 mg/ml) for up to 24 h at 25°C resulted in no decrease in E_{300} although yeast RNA added to the mixture was rapidly digested, as shown by a fall in E_{300} . If carried out at 0°C the same result was obtained, and the *o*-diphenol oxidase activity remained unaffected. Attempts to precipitate the RNA by adding manganese sulphate to a final concentration of 0.1 M or streptomycin sulphate to a final concentration of 5% (w/v), either at pH 5.5 or pH 9.5, failed to precipitate any RNA.

Fig. 4. Disc electrophoresis of purified (a) and crude (b) *o*-diphenol oxidase. The direction of electrophoresis is downwards. Samples (0.05 ml) in 0.05 M-tris-HCl buffer, pH 8.2, were applied to the gels (4% by 2%), which were made up in the same buffer. Electrophoresis was carried out for 1 h at 150 V and 3 mA/tube with 0.05 M-tris-glycine buffer, pH 8.2, in the reservoirs. The single band of protein in the pure preparation corresponded with the active band (for staining details see the text).

Attempts at removing the RNA by hydroxyapatite or DEAE-cellulose chromatography led to very low recovery of activity from the columns (<20%) and to the generation of up to four peaks of activity in either case. No inactive protein peaks were observed. The proportions of the four peaks varied considerably from one experiment to another and all four had much lower specific activities than the material applied. The peaks also had less RNA (up to 0.15 mg/mg of protein) associated with them. These experiments were abandoned because of the loss of activity and the lack of reproducibility. One feature of possible significance was that the last peak to emerge from hydroxyapatite columns always had the highest specific activity and the highest RNA content of the four.

Polyacrylamide-gel electrophoresis. A single band of protein, which corresponds to the active band, was observed when the purified enzyme was subjected to analytical disc electrophoresis at pH 7.8, 8.2 or 8.6, in 4% by 2%, or 7.5% by 5% gels, as shown in Fig. 4.

Effect of pH on enzyme activity. To study the activity of the purified enzyme in the pH range 3.5–8.5, the potassium phosphate buffer, pH 6.8, normally used in the assay procedure was replaced by a buffer prepared by mixing 0.1 M-citric acid with 0.1 M- K_2HPO_4 to give the required pH. Fig. 5 shows the resulting pH profile with a maximum at pH 5.0. At pH 6.8 where the activity was

normally measured, the activity is 66% of the maximal activity.

Substrate specificity. Although the natural substrate in the potato is probably chlorogenic acid, the enzyme catalyses the oxidation of 4-methylcatechol at approximately ten times the rate found with chlorogenic acid or catechol, the specific activities with these three substrates being 87.0, 9.0 and 8.4 U/mg respectively. The values were obtained in the routine assay with a substrate concentration of 0.26 mM in each case, which is sufficient to saturate the enzyme (Lavollay, Llegend, Lehongre & Neumann, 1963). For this reason, and because chlorogenic acid is an expensive reagent, 4-methylcatechol was used as the substrate for routine assays.

Fig. 5. pH profile of potato *o*-diphenol oxidase activity.

Copper content and minimal molecular weight. The protein component of the enzyme, after removal of RNA by trichloroacetic acid precipitation of the protein, was found to contain 0.17% of copper by weight. This indicates a minimal molecular weight of 36400 for the protein component, or 72800 for the RNA-protein complex.

Amino acid analysis. Four different samples of the enzyme were hydrolysed, two for 24 h and one each for 48 h and 72 h. In addition two more samples were oxidized with performic acid and then hydrolysed for 24 h to determine the half-cystine content. Because the weights of protein taken were not the same for each analysis, after each one the composition of the sample, in μ mol of each amino acid, was plotted against that of sample 1 (cf. Williams & Harrison, 1968), and the slope of the line was used to normalize the analysis with respect to sample 1. In determining the slope of the line the points for serine and threonine were disregarded as these amino acids decompose slowly during hydrolysis. Points for proline were also disregarded in measuring the slope of the line because proline was released only slowly from the protein.

The normalized values (μ mol) for each amino acid, except for serine, threonine and proline, were then averaged as given in Table 3, column 1. The normalized values for serine, threonine and proline were plotted against time and, in the first two cases, extrapolated to zero time to give the corrected average value. The plot for proline showed that its release was virtually complete after 72 h so this value was taken. Tyrosine usually decomposes slowly during hydrolysis while isoleucine and valine

Table 3. Amino acid analysis of the purified enzyme (six samples)

	Average number of μ mol $\pm$ s.d.	Average number of μ mol $\times 8.87$	Nearest integer
Cysteic acid*	0.055	4.9	5
Aspartic acid and asparagine	0.374 ± 0.007	33.2	33
Methionine†	0.071 ± 0.013	6.3	6
Threonine	0.193	17.1	17
Serine	0.251	22.3	22
Glutamic acid and glutamine	0.353 ± 0.009	31.3	31
Proline	0.190	16.9	17
Glycine	0.282 ± 0.011	25.0	25
Alanine	0.271 ± 0.012	24.0	24
Valine	0.234 ± 0.020	20.8	21
Isoleucine	0.191 ± 0.005	16.9	17
Leucine	0.317 ± 0.002	28.1	28
Tyrosine	0.118 ± 0.004	10.5	10/11
Phenylalanine	0.160 ± 0.009	14.2	14
Lysine	0.225 ± 0.005	20.0	20
Histidine	0.065 ± 0.019	5.8	6
Arginine	0.135 ± 0.007	12.0	12

* Corrected for 94% recovery after performic acid oxidation (Moore, 1963).

† Estimated as methionine sulphone after performic acid oxidation, or directly as methionine.

may appear slowly, but in this case they were not found to do so (see Table 3).

If the value for arginine is set equivalent to 12 residues per molecule, all the other amino acids give close to integral values except tyrosine whose value lies between 10 and 11 residues per molecule. In trying to decide the correct nearest integer value for tyrosine the following procedure was adopted. For each amino acid except tyrosine, the nearest integer value was divided by the average number of μmol . The average of the ratios obtained was 8.87 ± 0.16 (S.D.), representing a coefficient of variation of only 1.8%. The average number of μmol of each amino acid, including tyrosine, was then multiplied by 8.87 to give the values shown in column 2 of Table 3, from which the nearest integer values shown in column 3 of the table were obtained. The figure obtained for tyrosine, 10.5 residues per molecule, is the only one to which any doubt attaches. The significance of this finding is discussed below.

DISCUSSION

The isolation procedure described succeeds in its primary aim of providing the enzyme in good yield, with high specific activity, and without measurable tanning. The material is pure by the criteria of disc electrophoresis under a variety of conditions and the integral ratios of the amino acids present. The presence of half-cystinyl residues and the association of the enzyme with RNA have both been established. Many questions, however, still remain unanswered and some new ones have been opened by this work.

The multiplicity of active peaks seen in the Bio-Gel P-300 chromatogram (Fig. 2) undoubtedly arises, at least in part, from the dissociation of the largest species, as shown by rechromatography of the latter. One cannot, however, rule out the possibility that there are other contributions to the heterogeneity seen in Fig. 2. The existence of multiple forms of o-diphenol oxidases, and the interconvertibility of some of these, has been found before and is a subject of great complexity (Patil & Zucker, 1965; Jolley *et al.* 1969a; Jolley, Nelson & Robb, 1969b; Kertesz, 1966; Harel & Mayer, 1968; Fling *et al.* 1963). One unexplained anomaly in our results is the finding of a single band in disc electrophoresis at pH 7.8–8.6, whereas rechromatography on Bio-Gel P-300 at pH 5.6–7.0 gives three peaks. The difference in the pH range is unlikely to be the explanation, as gel filtration experiments were carried out at higher pH values during development of the isolation procedure, and no differences were seen.

The specific activity of the enzyme isolated by the method is probably greater than previously published values for the potato enzyme (Alberghina, 1964; Patil & Zucker, 1965; Abukharma & Woolhouse, 1966) although it is difficult to compare values obtained by other methods and using other substrates. The best specific activity obtained in this work was 120 U/mg at pH 6.8 with 4-methylcatechol as substrate, at 25°C. The values probably mean little in view of the rapid inactivation occurring in dilute solutions. One further complication in the use of specific activity as a guide, either to purification or to absolute purity, is the observation that the total activity recovered from

Table 4. Comparison of the amino acid compositions of o-diphenol oxidases from various sources

Data taken from: ¹ Fling *et al.* (1963); ² Jolley *et al.* (1969a); ³ Kertesz (1966); ⁴ this work.

Amino acid	<i>Neurospora</i> ¹	Mushroom ²	Mushroom ³	Potato ⁴
Cysteic acid	—	5	4	5
Aspartic acid + asparagine	31	35	34	33
Methionine	2	5	5	6
Threonine	16	20	24	17
Serine	32	15	13	22
Glutamic acid + glutamine	22	30	29	31
Proline	23	18	18	17
Glycine	19	23	23	25
Alanine	24	17	18	24
Valine	17	16	17	21
Isoleucine	9	15	14	17
Leucine	24	21	20	28
Tyrosine	12	11	10	10/11
Phenylalanine	17	15	13	14
Lysine	12	13	12	20
Histidine	6	8	6	6
Arginine	14	11	13	12
Tryptophan	8	8	11	—

Bio-Gel P-300 is greater than that applied (Table 2). Varga & Neujahr (1969) reported a similar increase in activity on purifying the enzyme from yeast and suggest that the reason is the removal of a putative inhibitor.

The amino acid analysis of the enzyme gives integral residue ratios within the limits of experimental error ($\pm 3\%$ coefficient of variation) in all cases except that of tyrosine. On the basis of 12 arginine residues per chain, there are almost exactly 10.5 residues of tyrosine present. One possibility is that the chain is actually twice as big, containing 24 arginine residues and 21 tyrosine residues. A comparison with the amino acid compositions of purified enzymes from mushroom and *Neurospora* (Table 4) makes this seem unlikely. Another possibility is that, although the enzyme is not 'brown', some of its tyrosine residues have nevertheless become oxidized during isolation. A small amount of dopa which elutes between leucine and isoleucine in the analytical procedure used for the amino acid determinations (Atkin & Ferdinand, 1969), could remain unaccounted for, but it is more likely that any tyrosyl residues that had become enzymically oxidized as far as dopa would be oxidized further to the highly reactive dopaquinone and would be undetected. This is considered the more likely explanation. The value for tryptophan has not been determined, but assuming a value of 8 per chain, by comparison with the mushroom and *Neurospora* enzymes, the minimal molecular weight of the protein component becomes 35300 as compared with 36400 on the basis of copper content. Considering an equal weight of RNA to be associated with the protein, the minimal molecular weight of the RNA-protein complex would lie near 72000. The complex elutes in the void volume of Bio-Gel P-300, which implies that its molecular weight is about 300000 or greater. It is possible, therefore, that the complex is actually composed of four protein units and an equivalent amount of RNA by weight, giving a total molecular weight of 290000.

The content of basic amino acids, lysine, arginine and histidine, is not unusually high (38 per chain) whereas aspartic acid and glutamic acid and their amides account for 64 out of the total of 308. To bind RNA one would expect the protein to have a preponderance of basic over acidic amino acids, but until the amide content of the enzyme is known, no conclusions can be drawn.

The content of hydrophobic amino acids (leucine, isoleucine, proline, valine and phenylalanine) is 31% of the total. According to Van Holde (1966) globular proteins may be classed as 'single chain' or 'multiple chain' according to whether their hydrophobic content is less than or more than 30% respectively. Proteins with about 30% hydro-

phobic residues are likely to exhibit association-dissociation phenomena. All the *o*-diphenol oxidases analysed so far lie on this dividing line, which may account for their interconvertible forms. The presence of so much RNA in the purified enzyme, apparently tightly bound and possibly necessary for enzymic activity, raises some interesting possibilities. It is not absolutely established that all the RNA present is actually bound to the protein. The presence of one or more species of RNA of similar molecular weight to the protein, that are neither attacked by ribonuclease nor precipitated by the common RNA precipitants, cannot be ruled out.

A possibly significant feature of the Bio-Gel P-300 chromatogram (Fig. 2) is that all the protein peaks, whether active or not, have RNA associated to a greater or lesser degree and the same remains true after treatment with ribonuclease. This suggests that a number of proteins present in the potato are intimately associated with RNA. In this context, another enzyme from potatoes that is also concerned with the metabolism of aromatic amino acids, L-phenylalanine ammonia-lyase, has a high E_{260}/E_{280} ratio (Havir & Hanson, 1968). Here again treatment with ribonuclease did not digest any RNA and so the authors concluded that RNA was not present. Arlinghaus & Polatnick (1969) have reported that RNA bound to the RNA polymerase complex is resistant to the action of ribonuclease, so there is already some ground for believing that RNA-protein complexes may resist the action of nucleases. The *o*-diphenol oxidase from broad-bean leaves (Robb, Mapson & Swain, 1965) has a high E_{260}/E_{280} ratio, although the enzyme isolated from mushrooms (Kertesz & Zito, 1962) does not. In the case of the broad-bean leaf enzyme the recovery of nitrogen in the form of amino acids after acid hydrolysis was low as compared with the total nitrogen present before hydrolysis. This could have been due to the presence of nucleic acid in that enzyme also.

Much work remains to be done to clarify the relationship between the RNA and the protein in potato *o*-diphenol oxidase, but this and other possible ribonucleoenzymes could serve as useful models for the way in which nucleic acids and protein interact in, for example, ribosomes and RNA viruses.

K. B. acknowledges with thanks the receipt of a grant from the Leche Trust. Our thanks are also due to Professor H. W. Woolhouse for his advice and to Professor W. Bartley in whose Department the work was carried out.

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