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PARTICULATE AND SOLUBLE FORMS OF *o*-DIPHENOL OXIDASE FROM POTATO TUBERS

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Abstract—A soluble and two different particulate forms of *o*-diphenol oxidase have been obtained from aged or fresh potato slices by differential and density gradient centrifugation The particulate enzymes were shown to sediment with microsomes and peroxisomes, respectively Over half the enzyme activity of aged slices was found to be particle bound, with approximately twice as much enzyme in the microsomes as in the peroxisomal fraction Very similar distribution patterns have been obtained with fresh potatoes, which have an *o*-diphenol oxidase activity approximately one-third that of aged slices

INTRODUCTION

THERE are numerous reports on investigations on the nature and properties of *o*-diphenol oxidase from potatoes Almost all deal with the soluble enzyme However, a high percentage of the *o*-diphenol oxidase activity of potato tubers is located in particulate fractions, according to Alberghina,[1] 25% of the enzyme is found in the microsomes These results have recently been confirmed,[2] and in addition another 25% of the enzyme was shown to sediment with the crude mitochondrial fraction, most of it is located in the peroxisomal fraction obtained by isopycnic sucrose gradient centrifugation.

This paper reports the results of a more detailed study of the distribution of *o*-diphenol oxidase among subcellular fractions from aged and fresh potato tubers

RESULTS

When potato tuber cells are broken in an isotonic medium, *o*-diphenol oxidase activity is found in the soluble and in different particulate fractions. Table 1 shows the distribution of the activity of the enzyme in fractions obtained by systematic differential centrifugation starting from aged or fresh slices With aged potatoes, 57% of the activity was found to be particulate. It is apparent that the *o*-diphenol oxidase-containing particles differ considerably in size Sucrose gradient centrifugation of the different fractions indicated that the distribution obtained by simple differential centrifugation was due to an incomplete separation of two kinds of particles When applied to sucrose gradients, the enzyme activity in the 3200 *g* pellet sediments with the peroxisome fraction (Fig 1) Under such conditions the activity of the 100,000 *g* pellet sediments with the bulk of the microsomes The 11,400 *g* and the 38,000 *g* pellets contain mixtures of peroxisomal and the microsomal fractions. Results obtained with fresh potato slices show that the total enzyme activity is lower than in aged slices but that the distribution among different fractions is quite similar (Table 1)

The experiments described below, except where noted, have been carried out with aged

[1] F A M ALBERGHINA, *Phytochem* **3**, 65 (1964)
[2] H RUIS, *Hoppe Seyler's Z Physiol Chem* **352**, 1105 (1971)

TABLE 1 DISTRIBUTION OF *o*-DIPHENOL OXIDASE ACTIVITY DURING DIFFERENTIAL CENTRIFUGATION

Fraction	Activity (U) Aged slices	Fresh slices
3200 *g* pellet	9 1	2 8
11,400 *g* pellet	7 4	3 5
38,000 *g* pellet	12 0	3 1
100,000 *g* pellet	18 3	6 5
100,000 *g* supernatant	35 2	12 3

50 g potato slices, preincubated as described in the text or fresh, were chopped for 10 min with 50 ml of grinding medium, and filtered through Miracloth The suspension was centrifuged for 7 min at 1000 *g*, and the pellet discarded This was followed by centrifugation at 3200 *g* (10 min), 11,400 *g* (10 min), 38,000 *g* (15 min), and 100,000 *g* (120 min) The resulting pellets were suspended in 0 05 M phosphate buffer, pH 7 5, and pellets and supernatant were dialysed against the same buffer overnight

slices. The addition of relatively high amounts (1 g/g potatoes) of insoluble polyvinylpyrrolidone resulted in somewhat lower, but nevertheless significant activity in the particulate fractions 19% of the total activity were detected in a 11,400 *g* pellet, 25% in the microsomal fraction

As mentioned above, *o*-diphenol oxidase from the 3200 *g* pellet sediments to the same

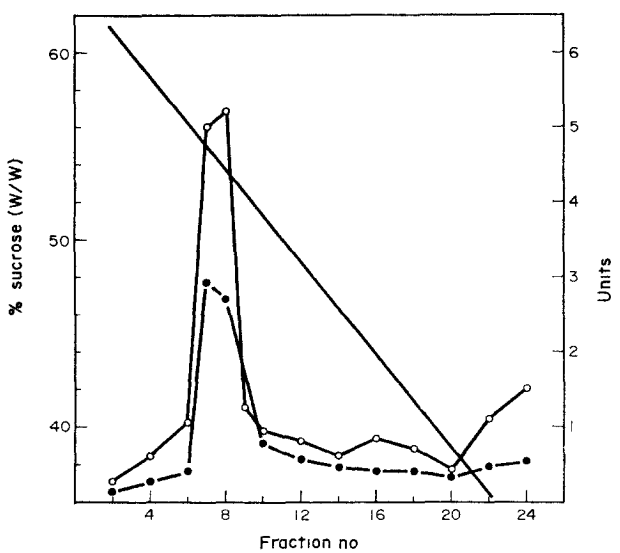

FIG 1 DISTRIBUTION OF CATALASE AND *o*-DIPHENOL OXIDASE ON LINEAR SUCROSE GRADIENTS The 3200 *g* pellet from differential centrifugation was layered on linear sucrose gradients (62–35%) The gradients were centrifuged in a Beckman SW 25 1 rotor for 5 hr and subsequently fractionated from the bottom Fractions were analysed for catalase and *o*-diphenol oxidase as described under Experimental ○—Catalase (μmoles H_2O_2 decomposed/min × ml ($\times 10^{-2}$)), ●—*o*-Diphenol oxidase (μmoles ascorbate oxidized/min × ml)

position in isopycnic sucrose gradients as catalase, a characteristic peroxisomal marker enzyme The position of the particle fraction in the gradient corresponds to a buoyant density of 1 26, which is close to the value of 1 25 reported for glyoxysomes[3] and leaf peroxisomes [4] The following enzymes, previously shown to be present in peroxisomes from other sources, have been detected in this fraction. catalase (specific activity 717 U/mg protein), urate oxidase (0 05 U/mg protein), malate dehydrogenase (14 5 U/mg protein), and glutamate oxalacetate transaminase (0 07 U/mg protein). The specific activities of these enzymes in the peroxisomal fraction are high enough—compared to activities in other subcellular fractions—to exclude the possibility that they arise from contamination Using fumarase as a mitochondrial marker enzyme, the peroxisomal fraction was shown to be virtually free of mitochondria or mitochondrial fragments Contamination of the fraction by soluble enzymes such as phosphorylase and phosphoglucomutase was also minimal (0 03% or less of the total activity of these enzymes) A more detailed characterization of potato tuber peroxisomes has been published elsewhere.[2]

Almost no *o*-diphenol oxidase activity was found in the second major organelle fraction in the gradient, the mitochondria, which sediment to approximately 43% sucrose (fraction 17, Fig 1) under the conditions used [2] The same distribution of *o*-diphenol oxidase on sucrose gradients was obtained with the 3,200 *g* fraction from fresh potatoes

When the pellet from the differential centrifugation was frozen and thawed once after resuspending it in grinding medium, neither a catalase nor an *o*-diphenol oxidase peak were obtained in the gradient region characteristic for intact peroxisomes Most of the catalase was at the top of the gradient and *o*-diphenol oxidase was almost evenly distributed throughout the whole gradient With osmotic shock treatment a similar result was obtained.

A number of experiments have been carried out to separate the *o*-diphenol oxidase activity from the peroxisomes The 3200 *g* fraction was layered on a linear gradient consisting of 10 ml 62% and 10 ml 50% sucrose, with 5 ml 35% sucrose on top of the continuous part of the gradient No separation of catalase and *o*-diphenol oxidase activities was achieved. In another type of experiment discontinuous gradients were used In one case these were prepared from 5 ml 62%, 10 ml 55 5% and 10 ml 50% sucrose Approximately 85% of the peroxisomes were found at the upper, 15% at the lower interphase In a second experiment 5 ml 62% 10 ml 54 5% and 10 ml 50% sucrose were used About half of the peroxisomal fraction was detected at the upper and half at the lower interphase. In both experiments, however, the ratio of catalase to *o*-diphenol oxidase activity was almost identical in both fractions The distribution of catalase and *o*-diphenol oxidase on isopycnic linear sorbitol gradients (30–60% sorbitol, 3 hr at 25,000 rev/min) was completely identical Sedimentation velocity experiments were carried out under various conditions Linear sucrose gradients (35–60%) were centrifuged for 15 and 30 min at 25,000 rev/min. Linear gradients from 15 to 30% sucrose were run for 15 min at 10,000 rev/min. In all these cases not even a partial separation of catalase and *o*-diphenol oxidase was obtained

Several attempts were made to solubilize the enzyme from the peroxisomal fraction. Neither osmotic shock, nor freezing and thawing, nor treatment with different concentrations of detergents like Triton X100 or deoxycholate did solubilize a significant part of the enzyme. After these treatments which solubilize most of the peroxisomal enzymes, still at least 80% of the *o*-diphenol oxidase activity was found in a pellet obtained by a 30-min centrifugation at 38,000 *g*.

[3] R W Breidenbach and H Beevers, *Biochem Biophys. Res. Commun.* **27**, 462 (1967).
[4] N E Tolbert, A. Oeser, R. K Yamazaki, R H Hageman and T Kisaki, *Plant Physiol.* **44**, 135 (196 9)

The microsomal (100,000 *g*) fraction was also subjected to sucrose gradient centrifugation In an experiment carried out under the same conditions as given in Fig. 1 most of the activity was found close to the top of the gradient (the maximum activity was detected in fraction 21) Essentially the same result was obtained with microsomes from fresh potatoes. When the 11,400 *g* and the 38,000 *g* pellets were centrifuged under analogous conditions, a 'microsomal' as well as a 'peroxisomal' *o*-diphenol oxidase peak were obtained

DISCUSSION

Before the discussion of the properties of particulate *o*-diphenol oxidase, the question has to be raised whether a part of this enzyme is really insoluble in potatoes or whether its isolation in particle fractions is due to an artefact According to Sanderson,[5] particulate *o*-diphenol oxidase from tea shoot tips can be obtained in soluble form when polycaprolactam powder is added to the extraction medium This seems to be due to the removal of polyphenolic substances which otherwise cause soluble proteins to precipitate

In potato tubers, however, there is no significant interference by polyphenols because of the following reasons:

(1) The type of effect described for tea shoot tips should be relatively unspecific with respect to different proteins For potato tubers it has been shown under conditions analogous to those used for experiments described in this paper that a number of enzymes remain virtually soluble during the preparation of the particle fraction [2]
(2) Addition of polyvinylpyrrolidone to the extraction medium did not change significantly the distribution of *o*-diphenol oxidase between soluble and particulate fractions

The results obtained show that a part of the *o*-diphenol oxidase is either associated with peroxisomes or with another particle not yet identified, which shows a behaviour very similar to peroxisomes during the separation procedures applied The association of the enzyme with the peroxisomes is somewhat surprising since it does not belong to the hydrogen peroxide producing oxidases which occur in these organelles For that reason various methods were tried to separate it from the peroxisomes But not even a partial separation of the enzyme activity from the peroxisomes was possible in isopycnic sucrose or sorbitol gradients of different composition, nor was separation obtained in sedimentation velocity experiments

In contrast to most peroxisomal enzymes, *o*-diphenol oxidase is not easily solubilized but seems to be firmly particle bound However, density gradient centrifugations after freezing and thawing or osmotic shock treatment show that the properties of *o*-diphenol oxidase-containing particles are altered considerably simultaneously with the solubilization of enzymes such as catalase from the peroxisomes The different behaviour of catalase and *o*-diphenol oxidase in these experiments should thus not be taken as evidence against the location of *o*-diphenol oxidase in peroxisomes This enzyme seems to remain associated with peroxisomal fragments when other enzymes are solubilized from the organelles At present no information seems to be available on other enzymes from plant peroxisomes that are firmly particle bound, but urate oxidase from rat liver peroxisomes is another example of a peroxisomal enzyme, which remains particulate after solubilization of most other proteins [6]

The microsomal *o*-diphenol oxidase particles are clearly different from peroxisomes The

[5] G W SANDERSON, *Biochem Biophys Acta* **92**, 622 (1964)
[6] F LEIGHTON, B POOLE, P B LAZAROW and C DE DUVE, *J Cell Biol* **41**, 521 (1969)

two fractions do not only sediment with different velocity but can also be easily separated on isopycnic sucrose gradients. Peroxisomal fragments with *o*-diphenol oxidase activity that are obtained by freezing and thawing or osmotic shock are different from the microsomal *o*-diphenol oxidase particles Whereas the microsomal particles are located in a distinct band on sucrose gradients the peroxisomal fragments are almost evenly distributed over a major part of the gradient. This fact seems to indicate that microsomal *o*-diphenol oxidase particles are not formed from peroxisomes during the preparation of the organelle fractions, but are of different origin

Most experiments described in this paper were carried out with aged slices since it has been reported that *o*-diphenol oxidase activity in potato tubers treated in this way is several times higher than in fresh ones.[7] A number of experiments were also performed with fresh slices. The results show the same distribution of *o*-diphenol oxidase in the aged and the fresh material

In summary, *o*-diphenol oxidase exists in potato tuber cells in at least three different environments, so that during isolation one soluble and two different particulate enzyme fractions are obtained The distribution of the enzyme in potatoes seems to be different from that observed in some other plants In tea shoot tips, all the *o*-diphenol oxidase activity has been reported to be soluble,[5] in grapes, the enzyme is completely particulate [8] Potato tubers seem to be between these extremes Whether this fact has any functional significance will have to be clarified by further work

EXPERIMENTAL

Extraction and preparation of fractions Potatoes, variety 'Allerfruheste Gelbe (Bohm)', were purchased on a local market Particulate fractions studied were generally obtained in the following manner peeled potatoes (50–200 g) were cut into slices, approx 1 mm thick, rinsed with tap H_2O and finally distilled H_2O until the washings were no longer cloudy The slices were moistened with distilled H_2O and aged by keeping them at 20° for 14 hr After another rinse with distilled H_2O they were chilled to 4° All subsequent operations were carried out at this temperature In some experiments fresh slices were used without preincubation After addition of grinding medium (1 ml/g 0 4 M sucrose, 0 165 M Tricine, 10^{-2} M KCl, 10^{-2} M $MgCl_2$, 10^{-3} M EDTA, 10^{-2} M dithiothreitol, and 0 2% bovine serum albumin, adjusted to pH 7 5) the potato slices were chopped with an onion chopper for 10 min Then the mixture was filtered through Miracloth and centrifuged for 7 min at 1000 *g*, the pellet was discarded A 15-min centrifugation of the supernatant at 3200 *g* gave a pellet containing the major part of the mitochondria and peroxisomes The pellet from a further centrifugation (15 min at 38,000 *g*) was discarded in most experiments The supernatant was now centrifuged for 2 hr at 100,000 *g* to obtain a microsomal pellet and a soluble supernatant

For isopycnic density gradient centrifugation the 3200 *g* pellet was resuspended in grinding medium and centrifuged over a linear sucrose gradient (62–35% sucrose) as described previously [2] Sucrose gradients were centrifuged in a Beckman SW 25 1 rotor in a Spinco L-50 ultracentrifuge Linearity of gradients was confirmed indirectly by measuring the distribution of dichlorophenolindophenol incorporated into them

Assay of enzymes The assay of *o*-diphenol oxidase was carried out by a modification of the procedure described by Balasingham and Ferdinand [9] The incubation mixture was 6 5 × 10^{-2} M phosphate buffer, pH 7 0, 2 7 × 10^{-4} M 4-methylcatechol, 3 8 × 10^{-5} M ascorbic acid, and 3 2 × 10^{-4} M EDTA Decrease of absorption at 265 nm was measured All enzyme assays were carried out in a total volume of 3 ml

Catalase was assayed spectrophotometrically at 240 nm as described by Luck [10] Urate oxidase was assayed in 5 × 10^{-2} M borate buffer (pH 9 0), with 6 6 × 10^{-5} M uric acid Disappearance of substrate was measured spectrophotometrically at 293 nm Malate dehydrogenase activity was measured at 340 nm in 6 5 × 10^{-2} M phosphate buffer (pH 7 5) with 1 2 × 10^{-4} M NADH and 5 × 10^{-4} M oxalacetate Glutamate oxalacetate transaminase was assayed in 10^{-1} M Tricine buffer (pH 7 5) The incubation mixture contained 3 × 10^{-3} M L-glutamate, 3 × 10^{-3} M oxalacetate, 8 × 10^{-3} M $MgCl_2$, 3 × 10^{-3} M NH_4Cl,

[7] E Marre, F Alberghina and O Servettaz, *Gazz Bot Ital* **69** V, 250 (1962)
[8] E Harel and A M Mayer, *Phytochem* **10**, 17 (1971)
[9] K Balasingam and W Ferdinand, *Biochem J* **118**, 15 (1970)
[10] H Luck, in *Methoden der enzymatischen Analyse* (edited by H U Bergmeyer), p 885, Verlag Chemie, Weinheim (1961)

1 1 × 10^{-4} M NADPH, 9 units of glutamate dehydrogenase, and enzyme The assay was carried out spectrophotometrically at 340 nm

Fumarase was assayed as described by Cooper and Beevers [11] Phosphorylase was assayed at 340 nm The incubation mixture consisted of 10^{-1} M phosphate buffer (pH 7 0), 8 mg soluble starch, 2 5 × 10^{-5} M D-glucose-1,6-bisphosphate, 10^{-2} M $MgCl_2$, 3 5 × 10^{-4} M $NADP^+$, 2 × 10^{-3} M EDTA, 10 units of phosphoglucomutase, 7 units of glucose-6-phosphate dehydrogenase, and enzyme solution Phosphoglucomutase activity was measured at 340 nm in 5 × 10^{-2} M Tricine buffer (pH 7 5), with 3 5 × 10^{-3} M D-glucose-1-phosphate, 5 × 10^{-5} M D-glucose-1,6-bisphosphate, 5 × 10^{-3} M $MgCl_2$, 3 6 × 10^{-4} M $NADP^+$, 2 × 10^{-3} M EDTA, and 7 units of glucose-6-phosphate dehydrogenase All enzyme activities are given in international units (U, μmoles substrate used up or product formed/min) Protein concentrations were determined by the method of Lowry *et al* [12]

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[11] T G COOPER and H BEEVERS, *J Biol Chem* **244**, 3507 (1969)
[12] O H LOWRY, M J ROSEBROUGH, A L FARR and R J RANDALL, *J Biol Chem* **193**, 265 (1951)

Key Word Index—Solanum tuberosum, Solanaceae, Potato, *o*-Diphenol oxidase Peroxisomes, microsomes