PURIFICATION AND CHARACTERIZATION OF O-DIPHENOLASE FROM WHITE YAM (D. ROTUNDATA) TUBER

BY

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DECLARATION

I declare that this work is wholly the author's and has not been part of any presentation for any other qualification.

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Sept. 25th 1981.

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DEDICATION

To Felix and uncle Felix.

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ABSTRACT

o-Diphenolases from ten cultivars of 4 cultivated Dioscorea species grown for food in Nigeria were studied as a result of the enzyme's importance in yam processing. The Km, Vmax and pH optima were different for the enzymes from the different cultivars. D. bulbifera$_n$ cv. Adu was found to contain the highest amount of 0—diphenolase activity with respect to catechol. The enzymes from all the cultivars showed maximum relative activity towards catechol, generally high or moderate activity towards other di- and polyphenols, and no activity towards monophenols.

The enzyme was purified from I), rotundata cv. Zaria to apparent homogeneity using a combination of ammonium sulphate fractionation, ion-exchange chromatography and gel filtration chromatography. Polyacrylamide gel electrophoresis revealed a single band which moved towards the anode at pH 9.0 as detected with catechol as substrate.

The pure homogenous enzyme was found to be a glycoprotein containing 24.2% carbohydrate, of molecular weight 107,333 + 5401, a copper Content of 0.22% (corresponding to 4 copper atoms per mole) and a pH optimum of 6.8. Of the inhibitors tested on the activity of the pure enzyme, sodium metabisulphite, DEDTC, DTT, cysteine, ascorbic acid, 2-mercaptoethanol, and potassium cyanide were found to be the most effective, while maleic hydrazide was the least potent. Inhibition by cysteine, 2-mercaptoethanol and potassium cyanide were found to be competitive with catechol as substrate, while NaN, showed uncompetitive inhibition.

EDTA and sodium chloride showed no inhibition. The heat of inactivavtion of the enzyme calculated from the Arrhenius equation was found to be 26,69 KcaL at pH 6.8 between 30 - 100^{o} C.

The UV absorption spectrum of the pure enzyme showed a peak at 280nm. The IR spectrum showed peaks at 3400 - 3300 cm^{-1} , 2100 cm^{-1} , 1650 cm^{-1} and at 1080 cm^{-1}.

From the effect of pH on Vmax, Km and —-— groups with pK values around 4.7 and 7.2 were identified to be involved in effecting transformation of substrate to product, binding of substrate to the enzyme and preservation of the native conformation of the active site.

The amino acid composition showed total absence of proline, cysteine and methionine.

INTRODUCTION

In many edible plant products, including the white yam, formation of several shades of colours generally termed "browning" occurs during handling, packaging and processing. The recognition of the enzymic nature of this type of browning in certain fruits was by Lindet as far back as 1895. The enzyme called o-diphenolase is known to be ubiquitous in the plant kingdom together with its phenolic substrates. In contrast with the various classes of non-enzymic browning, this type of browning is very rapid, intense, requires contact of the tissue with oxygen, and occurs only in fresh living tissues, or at least in tissues that contain active enzymes (Mathew and Parpia, 1971).

Most frequently, enzymic browning is viewed as an objectionable deterioration process which yields commercially undesirable products and therefore should be prevented, for example, in the production of banana puree, potato chips, yam flour and chips and in fruits and vegetables. On the other hand, enzymic browning is essential for the development of the desirable colour and flavour in the drying of tea, roasting of coffee, fermentation of cocoa, brewing of beer, and preparation of maple syrup (Mayer and Harel, 1979).

Thus the knowledge of o-diphenolase has been applied mainly in food processing, either to prevent browning or in facilitating enzyme reactions when desirable. o-Diphenolase enzymes are of great importance to man because the properties, palatability, and usability of most products derived from plants may be modified by the presence of the enzymes (Mayer and Harel, 1979).

White yam (Dioscorea rotundata) is easily the most widely cultivated species of yam and is second only to cassava as a source of calories for millions of people in Nigeria and West Africa. When the tuber is cut or bruised, the surface quickly turns dark brown, due to the presence of the enzyme o-diphenolase. This darkening affects the flavour, texture, aesthetic appeal and nutritional quality of white yam and its products, and constitutes a major setback in the use of the white yam for making "white" flour.

With growing sophistication of Nigerian consumers, the retention of original colour in processed yam products has become a matter of great importance to processors. Ordinarily, prevention of browning in vegetables during storage and processing has always been a challenge to food technologists. In the case of yam, processing is made even more difficult due to little or no information about the properties and effects of the browning enzymes during large-scale processing.

Although o-diphenolase has been partially purified from many plant tissues - tobacco leaf (Clayton, 1959); banana (Palmer, 1963); potato (Patil and Zucker, 1965); Clingstone peach (Wong et al, 1971); royal Ann cherries (Benjamin and Montgomery, 1973) and mango (Park et al, 1980), reports on purification to homogeneity, data on amino acid composition and copper content are relatively few. Most of the available information comes from fungi, particularly mushrooms and Neurospora, with only few reports from higher plants particularly those used for human food. In the case of Dioscorea species, only indirect reports on the presence of polyphenols associated with oxidative browning (Tono, 1976; Martin and Ruberte, 1976), and the transformation associated with o-diphenolase in Dioscorea alata during storage (Adamson and Abigor, 1980) have been published.

It is therefore important to initiate studies designed to systematically study the white yam o-diphenolase both in the isolated state and in the tuber itself. Such studies would lead to recommendations for an efficient storage of yam and the use of "white" yam flour with wheat flour in breadmaking, thus reducing the amount of wheat flour currently imported into Nigeria by the baking industry.

This will go a long way in conserving much needed foreign exchange. It is known, for example, that the products of the enzyme action (quinones) can react with free amino acids and amino acid side chains of proteins (Mathew and Parpia, 1971) in the white yam tuber thus preventing their availability and utilization in the body. So inactivating this enzyme would definitely make processed yam products much more nutritionally adequate. This investigation involving the purification and characterization of o-diphenolase from the white yam (Dioscorea rotundata) tuber will provide basic information about the properties and behaviour of the enzyme so vital in the storage and processing of the tuber.

LITERATURE REVIEW

In the early twentieth century, Bach and Chodat (see Joslyn and Ponting, 1951) proposed the view that the browning or darkening noted in injured plant tissues was caused by "oxygenases" which underwent autooxidation to yield a peroxide. According to them, this peroxide, with the help of peroxidase enzyme, brought about the oxidation of natural phenolic substances.

A major breakthrough was made by Onslow (1931), however, who found that almost all plant tissues known to darken on injury contained simple o-dihydroxyphenolic compounds - such as catechol and its derivatives, as well as hydroxy-gallic esters of caffeic acid such as chlorogenic acid - which are widely distributed in many plants. While it was found that such compounds did darken faintly on exposure, the browning was faster in the plant tissue itself when macerated. When the tissue was blanched, the browning reaction did not take place. Thus Onslow was able to modify the theory and considered as an enzyme the substance earlier designated oxygenase. She, however, considered the formation of peroxide to be a consequence of the oxidation of the dihydroxyphenol with molecular oxygen from air.



Meanwhile, it was demonstrated that browning reactions could take place in the absonce of peroxide (Szent-Gyorgyi, 1925). Subsequently, Nelson and Dawson (1944) proposed a theory of phenol oxidation in which the o-dihydroxyphenol was dehydrogenated leaving o-quinone and water as the end products.



Joslyn and Ponting (1951), used the term Polyphenol oxidase, abbreviated to polyphenolase or phenolase, to include all enzymes having the capacity to oxidize phenolic compounds to produce quinones.

Nomenclature:

The Commission on enzymes originally referred to enzymes capable of oxidising phenolic compounds as one of two types: (1) Catechol oxidase or o-diphenolase (O-DPO) or o-diphenol : O_2- oxido reductase (Ec.1.10.3.1). (2) Laccase or p-diphenol: O_2 oxidoreductase (Ec.1.10.3.2).

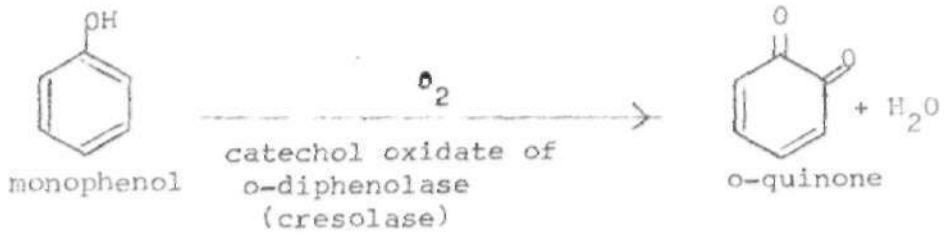
The Commission has now revised the nomenclature, lumping them together as Ec.1.14.18.1 - monophenol mono oxygenase. According to Mayer and Harel, (1979) this revised nomenclature is in many ways unfortunate because catechol oxidase which is also frequently referred to as Phenolase, polyphenoloxidase, tyrosinase, catecholase, o-diphenolase or cresolase, is quite distinct from laccase, and is specific mainly for o-diphenols. It does, however, oxidize monophenols in some cases. Laccase oxidizes a very wide range of substrates including monophenols, triphenols, and ascorbic acid as well as o-, m- and p-diphenols. The inability of o-diphenolase under any circumstances to oxidise p-diphenols is a typical property of the enzyme.

o-Diphenolase or catechol oxidase (Ec.1.10.3.1) and its distribution

The enzyme catechol oxidase thus belongs to class of enzymes known as oxidoreductase (EC.1), concerned with biological oxidation and reduction, and therefore with respiration and fermentation processes. They act on diphenols and related substances as donors (EC.1.10) with oxygen as acceptor (EC.1.10.3). These enzymes are otherwise called Phenolases (since they act on phenols) and has been variously named according to the substrates they act on, polyphenoloxidase, (polyphenols), monophenol oxidase (monophenols), tyrosinase (tyrosine) catecholase (dihydroxy phenol: oxygen oxido-reductase; EC.1.10.3.1).

According to Mathew and Parpia, (1971) catechol oxidase (o-diphenolase) is a copper enzyme which catalyses two distinct reactions.

(1) The insertion of oxygen in a position ortho to an existing hydroxyl group, usually followed by oxidation of the diphenol to the corresponding quinone, often called cresolase activity or hydroxylation activity

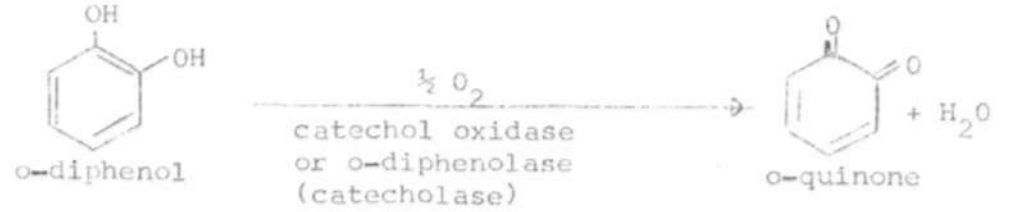


monophenol

o-quinone

and

(2) The oxidation, with hydrogen abstraction, of o-diphenol often referred to as catecholase activity, or dehydrogenation.



o-diphenol o-quinone

Molecular oxygen participates in both reactions. In food browning catecholase activity is more important than the cresolase activity since most of the phenolic substrates in food are dihydroxyphenols (Mathew and Parpia, 1971).

o-diphenolase is widely distributed, in the plant kingdom. Being easily detectable, it was among the first enzymes to be studied (Bertrand, 1895; 1896). It has since been reported to occur in several bacteria (Prabhakaran, 1968; Lerch and Ettlinger, 1972; Pomerenz and Murthy, 1974), numerous fungi, algae, bryophytes, pteridophytes, gymnosperms (Cambie and Bocks, 1966) and practically every order (and possibly family) of angiosperms where it has been looked for (Mayer and Harel, 1979).

All o-diphenolases so far investigated have activity on o-diphenols. o-Diphenolases from banana (Palmer, 1963), tobacco leaf (Clayton, 1959) tea (Gregory and Bendall, 1966), clingstone peach (Wong et al, 1971), royal ann cherries (Benjamin and Montgomery, 1973), cocoa husk (Chaplin, 1978) have been reported to have exclusively activity on o-diphenols and no ability to hydroxylate monophenols. o-Diphenolases from common mushroom (Smith and Krueger, 1962), potato (Patil and Zucker, 1965), apple, sugar beet leaf broad bean (Robb et al, 1966), and Triticum aestivum (Taneja and Sacchar, 1974) have both types of activity.

Certain isozymes of o-diphenolase from carrot and sorghum (Stafford and Dresler, 1972) and wheat (Taneja et al, 1974) have been reported to show only cresolase activity.

Subcellular location of o-diphenolase

o-Diphenolase is an intracellular enzyme (Mayer and Harel, 1979). Plant o-diphenolase has been located in a variety of cell fractions, both in organelles where it may be tightly bound to membranes and in the soluble fraction of the cell. Tanning reactions taking place after the disruption of tissues rich in phenols may cause binding of soluble o-diphenolase to a "particulate" fraction. Sanderson (1964) showed that the enzyme from tea leaves remains soluble if isolation is carried out in presence of polyamide, which adsorbs endogenous phenols. However,

Kato et al, (1976) reported that part of the activity in this tissue can be shown to be located in well defined particulate fractions even when phenols are removed with Polyclar AT.

In spite of possible artefacts, the weight of evidence indicates the wide occurrence of membrane-bound o-diphenolases, particularly in chloroplasts. Although many reports localizing the enzyme in organelles are based merely on differential centrifugation, some more careful studies involving density gradient centrifugation and correlation with chlorophyll content and activities of "marker" enzymes reached the same conclusion (Mayer and Harel, 1979).

Apart from its localization in chloroplasts (Harel et al, 1964; 1965; Harel and Mayer 1971), o-diphenolase has been reported to be localized in mitochondria (Bonner, 1955; Mayer, 1961) and in peroxisomes and "microsomes" (Ruis, 1972; Kato et al, 1976).

The strength of binding of o-diphenolase to membranes varies depending on the tissue and the stage of development of the plant. In some cases, drastic conditions are required for the solubilization of membrane bound o-diphenolase, such as the use of detergents, e.g. digitonin, Triton X-100, SDS, butanol or limited digestion with proteolytic enzymes (Harel et al, 1965; Alberghina 1964; Mayer 1966; Ben-Shalom et al, 1977).

These treatments evidently cause changes in the enzyme structure and/or conformation and are frequently accompanied by activation, as well as changes in the substrate specificity, pH optimum and other properties of the enzyme (Mayer and Harel, 1979). Solubilization of particulate forms of the enzyme to "soluble" ones occurs naturally during fruit ripening and aging as well as following exposure to "stress" conditions such as low humidity and deficiency in nutrients or growth substances e.g. in sugar beet leaves, apple, grape and olive (Harel et al, 1966; Ben-Shalom et al, 1977; Mayer and Harel, 1979).

Isolation and Purification of o-diphenolases

In view of the presence of many proteins of similar properties, the purification of o-diphenolase enzyme from plants is extremely difficult. This is further enhanced by the fact that the enzyme is generally present in very low concentrations. Kertesz and Zito (1962) estimated that even in the best mushroom samples (regarded as best source of the enzyme), the concentration of the enzyme is of the order of 0.004% (40mg/kg).

Another main problem in the isolation of o-diphenolase from plant tissue is the occurrence of the phenolic substrates and enzyme side by side. In living tissues, these two components are separated within the cells, but on maceration, extraction, and

other processing treatments, they come into contact and give rise to quinones and other condensation products. The products generally combine with the protein of the enzyme, making it inactive (Sanderson, 1965) with a resultant tanning. These tanning reactions taking place during the isolation of the enzyme result in changes in its properties as well as in apparent multiplicity (Bendall and Gregory, 1963). Some investigators have tried to prevent such reactions by "decolorization" with lead acetate, isolation under N_2 (Kertesz and Zito, 1965; Bouchilloux et al, 1963), or in the presence of reducing agents or phenol adsorbing agents such as polyethylene glycol (PEG) (Adamson and Abigor, 1980), polyamide or polyvinyl pyrrolidone (PVP) (Sanderson, 1964; Loomis and Battaile, 1966; Anderson, 1968; Benjamin and Montgomery, 1974). However, some of these agents inhibit the enzyme irreversibly (Walter and Hulme, 1965; Kidron et al, 1978).

In recent years this problem has been somewhat solved by the preparation of acetone powder as the initial step in isolation, permitting the interferring phenolic substrates to be removed from the enzyme source by extraction with cold acetone. Acetone has been preferred to alcohol, which is a better solvent for phenolic compounds occurring as substrates, because the former has been found to cause less inactivation of the enzyme.

The binding of o-diphenolase to membranes in many tissues further complicates its isolation. Solubilization is usually achieved after preparation of acetone powder or extraction with detergents and other agents (Mayer and Harel, 1979).

An additional obstacle to purification is the extensive multiplicity of the enzyme and the interconversions between forms which continually occur during purification steps and during storage of purified preparations (Mayer and Harel, 1979).

Procedures which have been employed in the purification of o-diphenolases include fractionation with ammonium sulphate, removal of inorganic impurities by dialysis, ion-exchange chromatography and gel-filteration. Celite has been found to be a selective adsorbent for o-diphenolases, being a relatively specific binder of copper proteins. Preparative isoelectric focussing has also been employed in some cases (Dubernet and Ribereau-Gayon, 1974; Kidron et al, 1978).

Attempts to purify o-diphenolases by affinity chromatography have had only limited success (Shimoda et al, 1975). This is apparently due to changed affinity of the phenolic ligands to the enzyme upon their binding to the support, to the oxidation of ligands during the operation of the column, and to the relatively low affinity of the enzyme for its phenolic substrates (Mayer and Harel, 1979).

Assay of o-diphenolases

Since oxidized phenolic substances undergo many secondary reactions both with each other and with protein, it is difficult to measure product formation in routine assays. It is also difficult to routinely assay for residual substrate since some of the products react with reagents for phenolic substances.

A reliable method of assay involves measurement of oxygen uptake directly using a Warburg manometer (Dawson and Maggee, 1955), taking only initial rates into consideration, since the enzyme may undergo rapid inactivation during catalytic performance. Bendall and Gregory (1963) found the Clark oxygen electrode extremely useful for measurement of the rate of oxidation of tea polyphenolases. However, the ratio of oxygen consumed/substrate oxidized changes with time of the reaction and depends on substrate type, concentration, pH of the reaction mixture and the buffer used (Mayer et al, 1966). An additional problem concerning this assay is the low affinity of phenoloxidase to oxygen. Most assays, and particularly the spectrophotometric ones are therefore carried out at oxygen concentrations far below Km for oxygen (KmO_2) of the enzyme. It is evident that true Vmax values are seldom determined (Mayer & Harel, 1979).

Methods have been proposed to follow o-diphenolase activity spectrophotometrically, using coupling reactions with the quinone formed. Ascorbic acid has been employed as a coupling reagent. The amount of ascorbic acid thus used up in the coupled oxidation, which is equivalent to the actual oxidation of the substrate, can be determined either by titration with dye (Sreerangachar, 1943) or spectrophotometrically (Ashraf El-Bayoni and Frieden, 1957; Mazzocco and Pifferi, 1976; Esterbauer et al, 1977). Good linearity with time and enzyme concentration was observed in both cases, but the exact stoichiometry of the reactions was not determined.

The most convenient and most widely used method is to follow the initial rate of formation of product spectrophotometrically (Patil and Zucker, 1965; Taneja and Bacchar, 1974; Benjamin and Montgomery, 1973; Interesse et al, 1980; Adamson and Abigor, 1980). The method is not only sensitive and rapid, but measures the initial oxidation of the substrate without including complex polymerization reactions or allowing for the inhibition of the enzyme by the products of the reaction.

Reaction Mechanism of o-diphenolase

The mechanism by which o-diphenolase reacts with its substrates is complicated by two problems. First, it is clear that the cresolase reaction involving apparently a hydroxylation reaction differs from the oxidation of the o-diphenols, the catecholase reaction. Secondly, it is not easy to follow the reaction of the copper, and the enzyme is also devoid of an absorption peak in the visible spectrum, so that the spectrophotometric analysis is also more complicated. It is however certain that copper is involved at some stage of catalysis, and there is the view that a pair of copper ions is required for oxidation of substrate - (Mayer and Harel, 1979).

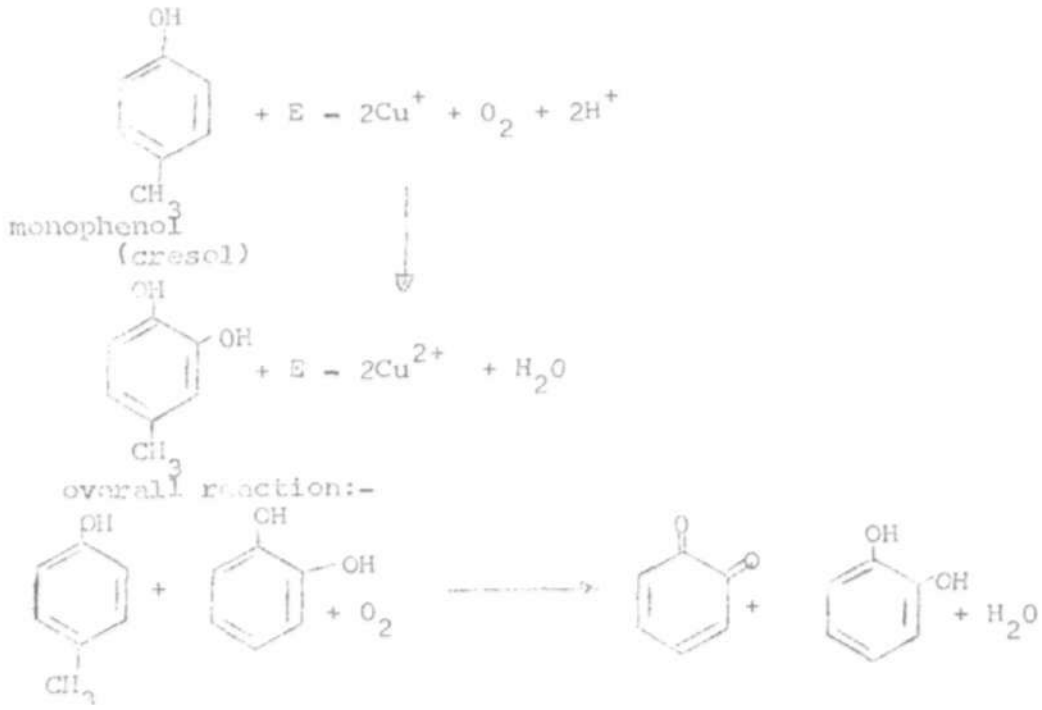
Mechanism of hydroxylation of Monophenols

For long, it was maintained by some authors that the hydroxylation reaction was non-enzymatic and only the dehydrogenation reaction was enzymatic. This view has been proposed by Onslow and Robinson, (1928) and vigorously advocated by Kertesz and Zito (1957; 1962; 1965). However, there is now overwhelming evidence against this view (Mason, 1956; Mathew and Parpia, 1971).

The onset of cresolase activity is often preceded by a lag period and electron donors, including o-diphenols, usually shorten or abolish this lag. The enzyme has to be "primed" or "activated" by an o-diphenol or by another reducing agent such as ascorbic acid or hydroquinone (Mason, 1956) before initiating the cresolase activity. Based on this observation and on the knowledge that o-diphenolase is a copper-metalloenzyme, Whitaker (1972) proposed the following reactions (where a small amount of catechol is added initially along with the monophenol)



The o-diphenol (catechol) functions to reduce the enzyme form E-2 Cu^{2+} to E- 2 Cu^{+} which can then hydroxylate the monophenol.



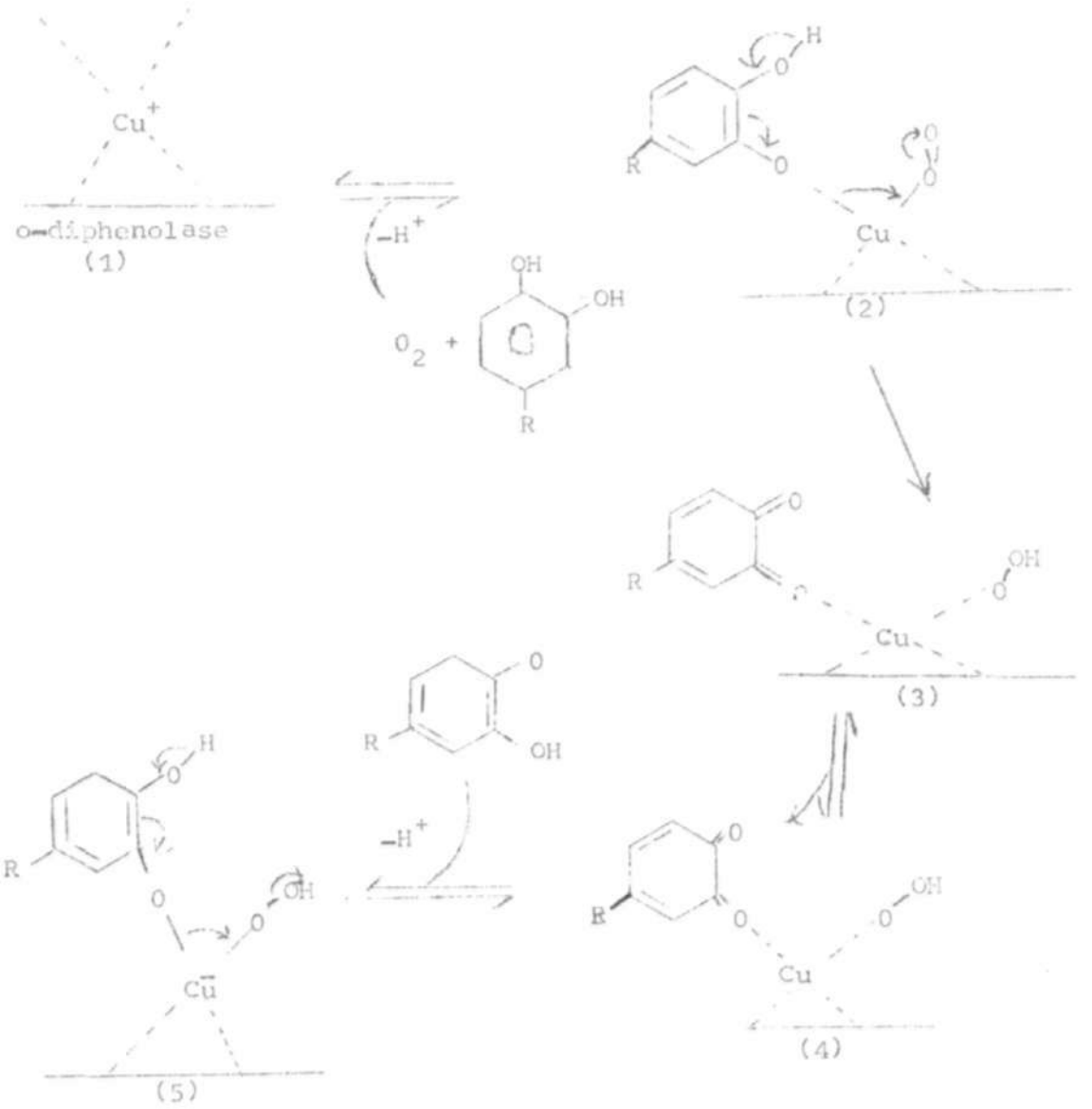
It is not clear whether the lag phase experienced without the o-diphenol is inherent in cresolase activity, or an artefact due to changes in the enzyme, during preparation and isolation (Mayer and Harel, 1979).

Mechanism of oxidation of o-diphenols (dehydrogenation)

The mechanism of oxidation of o-diphenols by o-diphenolase is not yet known with certainty (Mayer and Harel, 1979). Mason (1957) classified the catecholase activity as a four-electron transfer oxidase activity, in which oxygen is reduced to water or hydrogen peroxide without becoming fixed to the substrate. Thus, during the activity, two molecules of o-diphenols are oxidized to molecules of o-quinones, bringing about the reduction of one molecule of oxygen to two molecules of water.



Hamilton (1969) proposed a mechanism for the oxidation of o-diphenols by o-diphenolase; which shows the role of copper in this reaction.


Cu+
o-diphenolase
(1)
-H+
OH
OH
O2 +
R
H
O
R
O
O
O
Cu
(2)
O
R
OH
O
Cu
(3)
O
R
OH
-H+
H
O
R
O
O
OH
Cu
(5)
O
R
O
O
OH
Cu
(4)

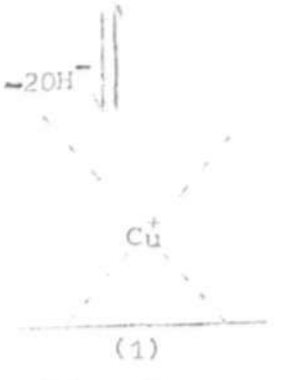


(6)





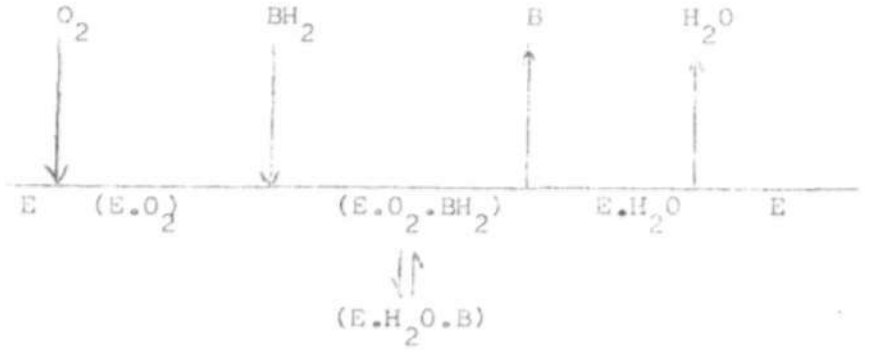
(8) (7)



(1)

o-diphenolase

Investigations of the order of binding of O_2 and o-diphenol (BH_2) to the enzyme (Ingraham, 1957), indicate that O_2 must bind first. The mechanism is thought to be an ordered BiBi mechanism.



The kinetics of o-diphenolase of grape chloroplasts has been studied by Lerner and Mayer (1976). The proposed sequence of binding of substrates is: first, random binding of an oxygen and an o-diphenol molecule followed by release of a product molecule prior to binding of the second o-diphenol molecule. The second product molecule is then released. In potato o-diphenolase O_2 is supposedly the first substrate to be bound to the enzyme (Mayer and Harel, 1979).

In considering the mechanism of reaction of o-diphenolase, the question of latency must not be forgotten. The changes in the enzyme which transform it from latent to active, are such that binding properties may change radically (Mayer and Harel, 1979).

There is a continuing argument on the physical relationships between the cresolase and catecholase functions of o-diphenolase. While some workers suggest that both functions are catalysed at a single site (Kubowitz, 1938; Mason 1956), others implied the participation of two sites, either on the same enzyme molecule (Dressler and Dawson, 1960; Shimao, 1962), or on different ones (Macrae and Duggleby, 1968).

Many preparations of o-diphenolase from plants are devoid of cresolase activity (Clayton, 1959; Palmer, 1963; Rivas and Whitaker, 1973) although it is difficult to assess whether these represent genuine "native" forms of the enzyme. The lability of cresolase activity is well known and it has been suggested that the loss of cresolase activity results from changes in the structure of the protein during its purification (Mallette and Dawson, 1949; Mason, 1956; Vaughan and Butt, 1969). Loss of cresolase activity on solubilization of membrane-bound catechol oxidase has been reported by Harel et al, (1965) and (1973), while Vaughan and Butt, (1969) observed a sharp decrease in cresolase activity upon aging. On the other hand, cresolase activity might be induced or increased after treatment with hormones, trypsin or urea or following infection (Mayer & Harel, 1979).

PROPERTIES OF o-DIPHENOLASE

1 Multiple forms

Reports on multiple forms of o-diphenolase started to flood the literature as soon as methods of protein fractionation such as ion exchange chromatography, gel filteration, gel electrophoresis and isoelectric focussing were introduced to study the enzyme. Most reports on o-diphenolase mention multiple forms or "isozymes".

Five phenolases were separated from mushrooms by chromatography on hydroxyapatite columns (Smith and Krueger, 1962). Four forms of mushroom phenolases (α-, β-, γ- and δ- forms) were also isolated by Bouchilloux et al, (1963). Some of these forms have been found to be interconvertible (Rolly and Mason, (1963). Kertesz and Zito (1965) obtained from mushroom a single form of active phenolase enzyme in a highly purified form. The reproducibility of the method, and the fact that the amino acid composition of some of the multiple forms obtained earlier was similar, lend support to the assumption that these multiple forms may be artifacts of a single active form.

An o-diphenolase preparation from potato peelings has been found to separate into two components on chromatography with DEAE-cellulose (Patil and Zucker, 1965). Also three components of o-diphenolase have been isolated from healthy sweet potato roots by Hyodo and Uritani, (1965).

From apples, four different o-diphenolases were found (Harel et al, 1965). While the possibility of the formation of artifact has been considered in mushroom, in this case the investigators are of the opinion that such a possibility is unlikely, especially in view of the consistantly different properties obtained for different forms in spite of repeated separation and repurification. It is probable, however, that each enzyme is bound to a specific site within its subcellular structure where it fulfills a specific role. Walker and Hulme, (1966) obtained two o-diphenolases extracted from apple peel by use of Triton X-100 and separated them into two fractions on DEAE-cellulose.

The discussion of multiplicity obviously includes arguments as to whether it is a 'native' genuine phenomenon or results from the release of membrane bound forms, partial denaturation, fragmentation, proteolysis, activation of latent forms, tanning reactions. Interconversions between forms apparently due to association-dissociation phenomena, have been reported for the enzyme from several sources.

Such interconversions, which occur spontaneously can be accelerated by factors such as ionic strength, concentration or dilution, the presence of certain ions, urea, and by presence of the phenolic substrate - (Mallete and Dawson, 1949; Smith and Krueger, 1962; Jolley et al, 1969; Harel et al, 1973; Mayer and Harel, 1979).

A great deal of attention has been paid to the differences in the properties of multiple forms of the enzyme and to the possible "physiological" significance of such differences. These include differences in affinity and specificity to phenolic substrates and oxygen (Harel et al, 1955), sensitivity to inhibitors, pH optimum (Wong et al, 1971; Kahn, 1976), and inactivation by heat (Ben-Shalom et al, 1977). Differences in "isozyme pattern" have been reported also in connection with subcellular organelles, the stage of development of the tissue and as a result of attack by pathogens or treatment with plant hormones (Harel et al, 1965; Taneja and Sacchar, 1977a; 1977b; Mayer and Harel, 1979). Although such differences in "isozyme patterns" might have a physiological significance, the establishment of such significance requires further evidence (Mayer and Harel, 1979).

Horowitz et al, (1961) have studied the genetic basis for the differences between isozymes in Neurospora crassa and their findings indicate multigenic control of 'tyrosinase' synthesis, but clearly points to one locus as the structure determining region. They concluded that the structural differences are determined by small, functionally unitary genetic region, the T locus. The four 'tyrosinases' appear to represent four different alleles at this locus.

2 pH optima of o-diphenolase

The effect of pH on the activity of the enzyme from many sources have been reported. The pH optimum of most of the o-diphenolases studied is between pH 5.0 and 7.0. However, survey of the literature reveals conflicting reports on the pH optimum of the enzyme from the same source e.g. in potato tubers (Patil and Zucker, 1965; Sharma and Ali, 1980) and in apples (Walker, 1964; Harel et al, 1965). There are also many reports on differences in the pH optimum, depending on the phenolic substrate being oxidized (Clayton, 1959; Alberghina, 1964; Robb et al, 1966; Vasmos-Vigyazo and Gajzago, 1978).

A feature which recurs in many reports is the presence of two peaks (Patil and Zucker, 1965; Adamson and Abigor, 1980; Intersesse et al, 1980; Sharma and Ali, 1980) in potato, Dioscorea alata, wheat and Solanum melongena o-diphenolases, or a wide optimum in the pH curve of enzyme activity (Paul and Janave, 1973; Ben-Shalom et al, 1977).

Changes in the form of the pH curve during development, or as a result of changes in growth conditions or treatments of the isolated enzymes with various agents have also been reported. These include changes in the pH optimum upon aging of tissue cultures, exposure of the tissue to stress conditions and treatment of the enzyme with denaturing agents such as detergents (Harel et al, 1965), and urea (Lerner et al, 1972), elevated temperatures (Kenten, 1958) or short exposure to acid pH (Lerner et al,1972). Such changes are sometimes accompanied by activation of the enzyme in the neutral to alkaline region of its pH curve. They might be explained by partial denaturation of the enzyme and/or conformational changes which result in shifts in its pH optimum. It is not unlikely that similar changes in the pH curve take place also in vivo during aging of the tissue, following release of membrane bound enzyme to the soluble fraction of the cell during fruit ripening or upon damage incurred by stress conditions (Ben-Shalom et al, 1977).

3 Substrate specificity of o-diphenolase

While o-diphenolases from animal tissues are relatively specific for tyrosine and dihydroxyphenylalanine - dopa (Mason, 1955), the plant enzyme acts on a wide range of mono-and o-diphenols. In addition, the specificity for optical isomers, which is clear cut in o-diphenolases from mammals is less evident in plant enzyme (Mayer and Harel, 1979).

It has been found that the rate of oxidation of o-diphenols by lettuce and mushroom o-diphenolases increases with increasing electron withdrawing power of substituents in the para position - $CH_3 > H > SCN > COCH_3 > CHO > CN > NO_2$ - (Mayer, 1962; Duckworth and Coleman, 1970). O-Diphenol substition at one of the positions adjacent to the -OH groups (e.g. by $-CH_3$ or an additional -OH) usually are not oxidized. Mayer, (1962) suggested that these positions should remain free for oxidation to take place.

The o-diphenol might be undergoing ketonization in the process of its oxidation by the enzyme (Mayer and Harel, 1979). Additional support for this suggestion may come from the observation that 2,3-naphthalenediol is not a substrate of o-diphenolase, but inhibits the enzyme competitively (Mayer et al, 1964). This compound cannot undergo keto-enol tautomerism and does not form a quinone due to fixation of the double bonds near the hydroxyl groups.

Although o-diphenolase can oxidize a wide range of phenolics, the individual enzyme tends to "prefer" a particular substrate or a certain type of phenolic compound. In some cases, the preferred substrate is also the most abundant phenolic in the particular tissue (Palmer, 1963; Challice and Williams, 1970).

o-Diphenolase of pear was found to act on a number of o-diphenols, but the activity was in descending order for catechol, chlorogenic acid, caffeic acid, and protocatechuic acid (Luh et al, 1963). Nakabayashi (1954), found that of all the o-diphenols tested, apple o-diphenolase had maximum activity towards hydrocaffeic acid, followed by chlorogenic acid, caffeic acid, 3,4 dihydroxy phenyl acetic acid and 3,4-dihydroxy benzoic acid. A number of substrates such as tyrosine, catechol, dihydroxy phenylalanine, pyrogallol, guaicol, resorcinol, hydroquinone, and p-cresol were oxidized by the action of o-diphenolase from sugar cane, while phenol and m-cresol were distinctly ineffective (Alexander, 1966).

Catechins in general have been found to be good substrates for tea o-diphenolases (Takeo, 1965), while quercetin and its glycosides were not appreciably oxidized (Robert and Wood, 1951). In the case of monophenols, Buruah and Swain, (1959) found that only those having para-substituted CH_2 groups such as p-cresol, tyrosine, and phloretin were active as potato o-diphenolase substrates.

The affinity of plant o-diphenolases for the phenolic substrates is relatively low. The Km is high, usually in the order of mM (Palmer, 1963 ; Harel et al, 1973; Rivas and Whitaker, 1973).

The affinity of o-diphenolases for oxygen is also relatively low, similar to other copper containing oxidases, and it depends on the phenolic substrate being oxidized, and could vary also among different forms of the enzyme isolated from the same tissue (Harel et al, 1965; Mayer and Harel, 1979).

4 Amino acid composition and Primary structure

Potato tuber, spinach beet and grape o-diphenolases have been reported (Mayer and Harel, 1979) to show considerable similarity in amino acid composition, both among themselves and compared to the fungal (mushroom, Neurospora and streptomyces) enzymes, and to silkworm and mouse melanoma tyrosinase. The similarity is particularly striking in the basic amino acids and in the content of threonine, serine, glycine, alanine and isoleucine.

A relatively low content of sulphur amino acids has been reported in o-diphenolases from various sources. Robb et al, (1965) found neither methionine nor cysteine in broad bean catechol oxidase while in potato tubers (Balasingam and Ferdinand, 1970) and grapes (Kidron et al, 1977), the enzyme did not contain methionine.

There are some reports on non-amino acid constituents in the structure of o-diphenolase. Balasingam and Ferdinand (1970) reported that RNA accounts for half of the weight of purified potato tuber o-diphenolase. The RNA could not be digested by RNase but its removal by other methods resulted in complete loss of enzyme activity. Yunusov et al, (1975) claimed that 26% of the enzyme from cotton consist of an arabinose, xylose, galactose and glucose-containing polysaccharide. According to them, the carbohydrate moiety is attached through the hydroxyl of a C-terminal serine. The N-terminal amino acid in the cotton enzyme is histidine.

In agaricus, isoleucine is the N-terminal while valine is the C-terminal amino acid. An N-terminal pentapeptide isolated from Neurospora o-diphenolase has the sequence N-Ac-Ser-Thr-Asp-Ile-Lys (Mayer and Harel, 1979). No work has been done on the amino acid composition and primary structure of o-diphenolases from Dioscorea species.

5 Molecular weight (MW) and quaternary structure

Lewis and Dawson (1948) reported that highly purified enzyme from Lactarius piperatus has a molecular weight of 100,000 which corresponds to four copper atoms per mole. The molecular weight of the predominant form of mushroom o-diphenolases has been often reported to be between 116000 and 120,000 daltons (Smith and Krueger, 1962; Bonchillou et al, 1963; Kertesz and Zito, 1965; Jolley et al, 1969)

The minimum molecular weight observed by sedimentation was 26000-32000 (Kertesz and Zito, 1957; Duckworth and Coleman, 1970). The enzyme was supposed to consist of four idential subunits (MW ca 30,000) each containing one copper atom.

This assumption was strengthened by observation of association-dissociation equilibra among the various forms, which were concentration dependent. Jolley et al, (1969) reported that the equilibrium could be shifted toward association by increasing protein concentration and by the presence of Ca^{2+}. Dissociation was facilitated by elevated temperatures, increased ionic strength, acid pH, and by the presence of SDS or EDTA..

Following the observation that copper atoms in mushroom o-diphenolase occur in pairs (Makino and Mason, 1973; Makino et al, 1974), Strothkamp et al, (1976) reinvestigated the quarternary structure of the mushroom enzyme. SDS-acrylamide gel electrophoresis showed that the enzyme contains two types of polypeptide chains, a "heavy" (H, MW 43000) and a "light" (L, MW 13400) chain, differing in their amino acid composition. It was concluded that the previously designated "monomer" of 26,000-34500 MW was a dimer of the L form, that the predominant form found in extracts (MW ca 120,000) was a tetramer of quarternary structure L_2H_2 and the active enzyme (apparent MW 69000) may then be L_2H, having two copper atoms.

The distribution of the copper atoms among the L and H subunits is yet to be determined.

The problem of MW and quarternary structure of higher plants o-diphenolase is more complex (Mayer and Harel, 1979). Most reports on the MW of the enzyme are based on estimates (e.g. by employing gel filteration or acrylamide gel electrophoresis) using partially purified preparations: the values reported cover a wide range. In many cases, crude or partially purfied preparations show multiplicity of forms which may have resulted from association-dissociation. Thus Harel and Mayer (1968) observed three forms of o-diphenolase from apple fruit having MWs of 30-40, 60-70, and 120-130K. These were shown to undergo interconversions. Demenyuk (1974) observed 24, 67 and 13K forms also in apple, the high MW one being transformed into the lower MW forms during ripening and storage of the fruit. Multiplicity of MWs has also been observed in o-diphenolases from tea leaves, avocado (Dizik and Knapp, 1970), potato tubers (Balasingam and Ferdinand, 1970), sugar cane (Coombs et al, 1974), banana and apple leaves (Heinaru et al, 1975).

o-Diphenolases which have been purified to homogeneity have MWs around 36-40K (Balasingam and Ferdinand, 1970; Vaughan et al, 1975; Kidron et al, 1977) with ca one gram atom copper per mole.

Mayer and Harel, (1979) suggested that this might represent the basic subunit of plant o-diphenolase. Gregory and Bendall, (1966) concluded that the native tea o-diphenolase has MW of 144 ± 16K, the multiplicity observed being the result of partial degradation and tanning reactions. Harel et al (1973) reported that the predominant form of grape o-diphenolase (55-59K MW) underwent "dissociation" upon storage, dilution or exposure to acid pH or urea, forming a 31-33 and 20-21K "subunits".

Does higher plants o-diphenolase also have a "heavy" and a "light" subunit? - is a question still to be answered. Mayer and Harel (1979) speculated that the 11700 MW form of the cotton enzyme and the 14K form from avocado might represent such a 'light' subunit, while values of 23500, 25K and 28K might be its dimers, and the frequently observed forms of 60-70K MW - the L_1H and L_2H combinations.

6 Copper content

Copper has been found to be present in small amounts in o-diphenolase preparations (Kubowitz, 1938) and is therefore considered its prosthetic group.

In several of their mushroom o-diphenolase preparations, Kertesz and Zito (1965) found that all the copper was in the cuprous state after testing with 2,2'-diquinoyl immediately after the preparation. Robb et al, (1965), Kertesz and Zito, (1965), Vaughan et al, (1975) and Kidron et al, (1977) reported a copper content of one atom per polypeptide chain or subunit in broad bean, mushroom and sugar beet o-diphenolases, respectively. An earlier report by Gregory and Bendall, (1966) suggest the presence of more than one copper atom per polypeptide chain. Kertesz et al, (1972) suggested that two non-equivalent copper atoms are present per catalytic site of mushroom o-diphenolase. However, recent observations (Mayer and Harel, 1979) with Agaricus and Neurospora o-diphenolases indicate that the functional unit of the enzyme contains a pair of copper ions, and raises the question as to whether such a pair is attached to a single polypeptide chain or to two identical or different subunits.

Determination of the copper content of o-diphenolase is complicated by the apparent loss of copper from the enzyme during its purification (Kertesz and Zito, 1965; Jolley et al, 1969; Duckworth and Coleman, 1970; Kidron et al, 1977).

Although the presence of copper in o-diphenolase is not disputed, and its participation in the oxidation is well established, just what happens with the copper is not adequately known. The copper in the resting enzyme can apparently be either oxidized or reduced or bound to peroxide (Mayer and Harel, 1979).

7 Latency, Activation and Induction of o-diphenolase

Latency and activation of plant o-diphenolases were first studied by Kenten, (1957) in broad bean (Vicia faba) leaves. Activation was achieved by short exposure to acid (pH 3.0 - 3.5) or alkali (pH 11.5). The activation was ascribed to the removal of an inhibitory protein which was assumed to be attached to the enzyme. Activation by anionic detergents was interpreted to act by combining with cationic groups of the enzyme which results in dissociation of the "enzyme-inhibitor complex" (Kenten, 1958; Yamaguchi et al, 1970). Swain et al, (1966) interpreted the activation of broad bean o-diphenolase by detergents and short exposure to acid, alkali or urea as involving a limited conformational change rather than dissociation or aggregation. Conformational changes as the cause of activation were also suggested by Lerner et al, (1972).

The activation was due primarily to an increase in V_{max} while affinity for the phenolic substrate decreased and that for oxygen increased. The process was reversible and could be repeated several times with the same preparation. Conformational changes caused by a long exposure to pH 2-3 were observed also in purified mushroom o-diphenolase (Duckworth and Coleman, 1970). Latency due to combination of the enzyme with a diffusable inhibitor was suggested in spinach chloroplasts (Sato and Hasegawa, 1976).

In tomato (Abutalybov et al, 1956), potato (Petrova, 1963; Gedz, 1965), apple (Pelgomen, 1964), cotton (Aliev, 1965), oat (Rubin et al, 1965), trace elements such as manganese, copper, and boron were found to increase the activity of o-diphenolase. Exposure to electric current increased o-diphenolase activity in apple and pear (Janikov, 1961), while γ-irradiation of banana fruits increased browning and activity of the enzyme in the skin (Chachin et al, 1965).

Induction of o-diphenolase due to de novo synthesis of the enzyme has been extensively studied in Neurospora by Horowitz and coworkers (1960, 1961 and 1970). The enzyme which normally appears in the reproductive phase of the life cycle, could be induced when vegetative growth was inhibited by starvation, metabolic inhibitors, or inhibitors of nucleic acid and protein synthesis such as actinomycin D, puromycin as well as amino acid analogues.

Synthesis of the enzyme has also been suggested to be involved in the 'induction' of o-diphenolase in germinating wheat seeds (Taneja and Sachar, 1977) which was affected by actinomycin D or cycloheximide.

8 Inhibitors of o-diphenolase

Inhibitors of o-diphenolase activity can be grouped into three classes: (1) those that are oxidized in preference to the substrate or resemble its structure (2) those that make the enzyme ineffective by interacting with the prosthetic group-copper and (3) those that complex with the substrate making it unavailable to the enzyme.

Ascorbic acid has been found to be effective in controlling enzymic browning by becoming oxidized itself (Mathew and Parpia, 1971). Competitive inhibitors of the phenolic substrates include compounds which are slowly oxidized substrates e.g. 4-nitrocatechol, p-nitrophenol, 4-chlorophenol (Clayton, 1959; Robb et al, 1966; Lerch and Ettlinger, 1972) and compounds which are not oxidizable but resemble the substrate's structure e.g. benzoic acid, other aromatic carboxylic acids and 2,3-naphthalene diol (Mayer et al, 1964; Harel et al, 1965; Duckworth and Coleman, 1970; Rivas and Whitaker, 1973).

Inhibitors which act on the copper in the enzyme include cyanide, diethyl dithiocarbamate and thiourea derivatives such as phenyl thiourea. Other metal ion chelators, less specific for copper also inhibit the enzyme in some cases - sodium azide and EDTA (Clayton, 1959; Wong et al, 1971). However, o-diphenolase copper is sometimes relatively inaccessible to inhibitors (Mayer and Harel, 1979).

Polyvinylpyrrolidone (PVP) and polyethylene glycol (PEG) adsorb phenolics and have been widely used to protect plant enzyme from inactivation by phenolics and their oxidation products (Loomis and Battaile, 1966; Anderson, 1968). Hulme et al, (1964) suggested that PVP inhibits o-diphenolase by combining with an o-diphenolase-substrate complex, probably by attachment to the phenolic substrate moiety of such a complex.

The interpretation of the mode of inhibition of o-diphenolase by various compounds is complicated in relation to the cresolase and catecholase functions. Differential inhibition of the two activities by some compounds has been interpreted to indicate separate sites for mono-and diphenols (Mayer and Harel, 1979).

Walker (1975) reported that cinnamic acid derivatives inhibit catechol oxidase (o-diphenolase) by acting on a specific "inhibitor site" on the enzyme.

Inhibition by SH-reagents is not a general phenomenon probably because o-diphenolases usually have low content of cysteine (Mayer & Harel, 1979). Inhibition by sulfhydryl compounds is effected by removal of the quinones from further participation in secondary browning reactions. Thiols such as cysteine and other thiol amino acids, mercaptoethanol, and amides of β-aminoethyl mercaptans, act as inhibitors by addition to the quinones and/or by being reducing agents (Mathew and Parpia, 1971).

There are several reports in the literature on "natural" inhibitors of o-diphenolase which have been isolated or detected from various tissues. A low molecular weight compound with an absorption peak at 280-290nm isolated from cultures of Penicillium expansum inhibited apple and tobacco enzymes (Walker, 1970). A short polypeptide isolated from Agaricus hortensis competitively inhibited the enzyme from the same tissue. Also a protein (MW 34000) isolated from potato tubers strongly inhibited cresolase activity of the enzyme from the same source (Mayer and Harel, 1979).

Not much work has been done on the inhibition kinetics of o-diphenolase from Dioscorea species.

Physiological Importance of o-diphenolase

In ascribing a physiological function to o-diphenolase in green plants, a number of properties of the enzyme must be taken into account; (1) its subcellular location, (2) the phenomenon of latency; (3) activation of the enzyme under certain conditions due to conformational changes; (4) the presence of native inhibitors; (5) the enormous variation in enzyme level at different periods of growth and development; (6) the separation of the enzyme from most of its substrate due to compartmentation, cellular or subcellular. A possible interpretation of these very diverse properties is a formidable task (Mayer and Harel, 1979).

One of the oldest suggestions of a physiological role is that of synthesis of o-diphenols in vivo (Mason, 1956). This suggestion is based on the undoubted ability of many o-diphenolase preparations to oxidize monophenols to the corresponding o-quinones. It is however not certain whether the o-diphenol is released as a distinct product or not.

A second long suggested role of o-diphenolases is in electron transport. Since quinones are powerful oxidizing agents it is easy to imagine a reaction in which a diphenol is oxidized by o-diphenolase to the corresponding quinone which then reduces some cell constituent, such as a nucleotide non-enzymatically.

Kubowitz (1938) claimed to have demonstrated such a reaction and since then o-diphenolase has been suggested to be involved one way or another in aerobic respiration (Crawford, 1967; Mayer and Harel, 1979).

It has been found that o-diphenolase activity or rate of darkening of tissues is proportional to resistance of the tissues to pathogenic bacteria, virus and fungi. Thus, o-diphenolase has been implicated in the resistance of plants to various micro-organisms because it oxidizes their phenols rapidly to quinones which kill the damaged cells, and produce a layer of dead tissue between the infected and healthy areas in sugar beet, potatoes, yams, bananas and damaged parts of stems or roots, which prevent the penetration of these micro-organisms (Daverall and Wood, 1962; Sisakian, 1963). Moustafa and Whittenbury, (1970) also observed that inhibition of o-diphenolase increases infectivity and decreases resistance.

The importance of quinones in vegetable tanning has been emphasized by Enders, (1961). He concluded that the essential reaction in vegetable tanning is 1,4-addition of free amino groups of the protein to low molecular weight quinones formed by the oxidation of phenols (by o-diphenolase), resulting in covalent cross-linkage.

The involvement of o-diphenolase in affecting the regulation of plant growth has been implied in various ways. Gordon and Paleg, (1961) demonstrated that quinones can interact with tryptophan to form, ultimately, indole acetic acid (IAA). They suggested, however, that the reaction occurs only in wounded tissues.

A new unexpected role of o-diphenolase may be in reactions which render seed coats impermeable to water. In seeds in which the enzyme is absent, the coats are permeable, while its inhibition prevents darkening and loss of water permeability. (Marbach and Mayer, 1975).

o-Diphenolases have also been reported to be involved in the formation of fruiting bodies in fungi, dopa and melanin synthesis, and its involvement in the biosynthesis of specific metabolite has also been implied, e.g. in the synthesis of the actinomycin chromophore (Mayer and Harel, 1979).

Evolution of o-diphenolase

o-Diphenolase is a very ancient enzyme. It is clearly present in prokaryotes e.g. streptomyces, Mycobacterium, and Bacillus subtilis, Pseudomonas melangenum, and Vibrio tyrosinaticus (Prabhakaran, 1968; Lerch and Ettlinger, 1972; Pomeranz and Murthy, 1974). In eukaryotes, it is almost ubiquitous (Mayer and Harel, 1979). In general, the enzyme reported in bacteria and fungi is very similar to that reported in higher plants.

Molecular weight and copper content show the same kind of characteristics although substrate specificity seems to be very variable. Too little is known about its subcellular location in the lower organisms to permit comparison with higher plants. The impression, however, is that in bacteria and fungi, the enzyme is primarily soluble and in the higher plants much of it is particulate. It is difficult to draw evolutionary conclusions. A ubiquitous enzyme of unknown physiological function present apparently in all stages of evolutionary development, must confer some kind of survival benefit to the organism in which it occurs. Perhaps the only trend which can be observed in the enzyme's properties is the decrease in its hydroxylating activity in higher plants. This perhaps is an indication of its changing physiological function during evolution (Mayer and Harel, 1979).

Yam (Dioscorea species)

Within the genus Dioscorea, the most important species economically are D. rotundata (white yam), D. alata (water yam), D.cayenensis (yellow yam), D. esculenta (chinese yam), D. dumentorum (bitter yam), D. bulbifera(aerial yam). D. rotundata is the most widespread species in Nigeria. It is grown in all parts of the tropics where growing conditions are suitable.

Several cultivars of D. rotundata exist. There are no universal names for the cultivars, but each locality has its own unique series of names for the different cultivars. Within each locality or linguistic group, there is no confusion. The traditional farmer is an expert in identifying and distinguishing between different cultivars of D. rotundata and of yams generally.

Ethno-agricultural importance of yam

In many parts of Nigeria, a considerable amount of ritualism has developed around the production and utilization of yams. One of the most important manifestations of this ritualism is the new-yam festival. Essentially a date is fixed for the festival, and before that date, no farmer may harvest or consume the new crops of yams. On the appointed day, each farmer harvests several yams from his farm, uses part of the harvest to prepare a feast, while the remainder may be used to pay homage to his elders or friends or in-laws. Considerable religious activity and social merry-making often accompany the new-yam festival. After the festival, the farmer may harvest the rest of his crop at his own pace and without further restriction. The fact that yams alone have a festival indicates that they occupied a singular position in traditional agriculture of earlier times.

Utilization of yam for food

By far the largest proportion of yams produced annually is marketed as the fresh tuber. Only a very small fraction goes to the market in processed forms.

Boiled yam is the simplest and commonest form in which yam is consumed. Its preparation involves boiling pieces or slices of yam in water.

Pounded yam is perhaps the most popular and most traditional form in which yam is eaten in Nigeria. It is prepared from boiled yam which is pounded in a large mortar until it forms a thick paste of uniform consistency. The exact consistency that results depends on the species or cultivars of yam being used. D. rotundata and D. cayenensis usually make good pounded yam, but even within these species, there are cultivars that are more suitable than others.

"Amala" This is made from yam flour. Amala is brown due to the oxidation of phenolics in the yam by o-diphenolase during the preparation of the flour. Upon reconstitution of the brown flour in hot water, the resulting paste is almost black but delicious when eaten with a suitable vegetable soup.

Other forms in which yam can be consumed are mashed yam, fried/roasted yam, fried yam-balls and yam flakes.

Yam tubers make good feed for livestock, but they are not normally used for that purpose, because of availability of much cheaper alternatives. In rural settings, yam peels are often fed to domestic animals such as goats and sheep.

Many species of yam contain small amounts of sapogenins and alkaloids for which various uses have been found. The main sapogenin present is diosgenin which serves as a starting point for the pharmaceutical manufacture of several corticosteroid drugs. The alkaloids present in yams include dioscorine and dihydro-dioscorine. Both substances are nerve poisons and the species that contain them are therefore used as sources of poisons for hunting, fishing, and pharmaceutical purposes (Onwueme, 1978).

EXPERIMENTAL

MATERIALS

Yam samples: Ten cultivars representing four species of yam grown during 1980 in different parts of Nigeria were used for this study.

Species	Cultivars	where purchased
D. rotundata	Zaria	Samaru-Zaria
	Amara	" "
	Orhohozar	Benin-City
	Omi	" "
	Obiaoturugo	Umudike, Umuahia
D. alata	Igiehua	Benin City
	Mbala	Umudike, Umuahia
D. cayenensis	Oku	" "
	Ikpen	Benin City
D. bulbifera	Adu	Umudike, Umuahia

Chemicals: Sephadex G-200 and DEAE-Sephadex A-50 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). The compounds tried as substrate for o-diphenolase were catechol, DL-3, 4-dihydroxy phenylalanine (dopa), pyrogallol, chlorogenic acid acid (supplied by BDH chemicals, Poole, England), as well as 3,4,5-trihydroxy benzoic acid, p-cresol, tyrosine, p-coumaric acid, and quinol (supplied by Hopkins and Williams, Chadwellheah, Essex, England)

The marker proteins - catalase, bovine serum albumin (BSA), D(-) lactate dehydrogenase were obtained from Sigma Chemical Company (St. Louis, U.S.A.) and egg albumen, cytochrome C, haemoglobin were from BDH Chemicals (Poole, England).

Acrylamide, N,N'-methylene-bisacrylamide (BIS), ammonium persulphate, and N,N,N',N'-tetramethylethylenediamine (TEMED), bromophenol blue, coomassive brilliant blue (all especially purified for electrophoresis) were obtained from Shandon Southern Products Ltd. (Runcorn, Cheshire, England).

Other chemicals used were either products of BDH Chemicals (Poole, England), or Hopkins and Williams (Chadwellheath, Essex, England), or Mayer and Baker (Dagenham, England), or Cambrian Chemicals (Beddington, Croydon), or Sigma Chemical Company (St. Louis, U.S.A.).

All reagents used (with the exception of sodium chloride, hydrochloric acid, ammonium sulphate, sodium carbonate, sodium hydroxide, sodium metabisulphite, potassium sodium tartrate and cupric sulphate which were laboratory reagents) were of analytical or pure grade.

Instruments:

The UV spectrophotometer used was the Beckman double beam DB-GT grating spectrophotometer.

High Speed Refrigerated Centrifuge - Beckan model J-21.

pH meter - corning model 7.

Atomic Absorption Spectrophotometer - Pye Unicam SP 1900.

Lyophilizer - Edwards Modulyo freeze dryer.

Infrared (IR) Spectrophotometer - Perkin-Elmer IR Spectrophotometer model 710B.

Weighing balance - Mettler Type H6T No. 215552.

DC Power Supply for Electrophoresis: Bio-Rad. Laboratories Model 500 Power supply.

Amino acid analyzer - Technicon TSM Sequential multisample Analyzer for amino acids.

METHODS

Preparation of 'Acetone powder' from yam tubers

The yam tuber was peeled and cut into pieces. The pieces were macerated in a Waring blendor with chilled acetone. The homogenate was then squeezed through two layers of cheese-cloth. The residue was spread on a flat surface inside a freezer to dry. The resulting dry powder called "acetone powder" was then bottled and stored in a freezer until used. This was done for each of the

Preparation of crude o-diphenolase (o-DPO) extract from acetone powders of yam cultivars

Ten grams of acetone powder from each cultivar was suspended in 30mls of 0.1M potassium phosphate buffer of pH 6.8, allowed to stand in an icebath for 1 hour with constant stirring, and then centrifuged at 20,000g for 10 minutes at 5°C. The supernatant which served as the crude enzyme was stored in a freezer until used.

Extraction and purification of o-diphenolase from white yam (D.rotundata cv. Zaria) tuber

o-Diphenolase from D. rotundata cv. Zaria was selected for further purification as described below:

All operations were carried out either at 4°C or with samples immersed in icebaths unless otherwise stated. All solutions were cooled before use. o-Diphenolase activity and protein content were determined after every step. This was to make sure that the enzyme protein being looked for was still present at every stage of purification.

Preparation of crude enzyme from acetone powder

Eighty grams of acetone powder was suspended in 240mls of chilled 0.2M potassium phosphate buffer of pH 7.5, and allowed to stand for 1 hour in an icebath with continuous stirring using a magnetic stirrer. Centrifugation of the slurry at 20,000g for 10 minutes at 5°C resulted in a very viscous and slimy supernatant fluid which formed the crude enzyme extract.

Ammonium sulphate fractionation

Preliminary work had shown that most of the enzyme activity is precipitated between 20% and 85% ammonium sulphate saturation (Appendix I, Fig.1).

Enough solid ammonium sulphate was added to the crude enzyme extract to give 20% saturation. The suspension was allowed to stand for 30 minutes in an icebath with constant stirring, and then centrifuged at 25,000g for 10 minutes at 5°C. The supernatant which contained most of the enzyme activity was brought to 85% saturation by addition of more solid ammonium sulphate. This was again allowed to stand for 30 minutes with constant stirring and then centrifuged at 25,000g for 10 minutes at 5°C. The resulting precipitate was redissolved in 21ml of 0.1M potassium phosphate buffer of pH 6.8, yielding about 33ml of enzyme solution. This is subsequently referred to as ammonium sulphate precipitated enzyme.

Dialysis

Thirty millilitres of the ammonium sulphate precipitated enzyme was dialysed for 2 hours against 0.1M potassium phosphate buffer of pH 6.8 at 4°C. The buffer solution was constantly stirred with a magnetic stirrer and was changed four times during the period of dialysis. The total volume of the dialysate was found to be 34ml.

Ion-exchange chromatography

Ten grams of DEAE-sephadex A-50 (capacity 3.5 ± 0.5 meq g^{-1}) was soaked in 0.1M potassium phosphate buffer of pH 6.8 for 1½ days at room temperature to swell. After removal of the fines and degassing the swollen DEAE-sephadex was poured into a glass column (2.6 x 40cm). The packed column was equilibrated at 4°C by pumping 0.1M potassium phosphate buffer of pH 6.8 through the column at the rate of 30ml per hour, until the pH of the effluent read 6.8. Thirty-two millilitres of the dialysed ammonium sulphate precipitated enzyme was then applied to the DEAE-sephadex column and allowed to drain into the column. The column was then eluted with a linear gradient from 0.1M potassium phosphate buffer of pH 6.8 to 0.5M NaCl in the same buffer. The flowrate was maintained at 30ml per hour. Ten millilitre fractions were collected with an LKB automatic fraction collector. Absorbance of fractions was monitored at 280nm with a Beckman DB-GT spectrophotometer to follow protein elution. The A_{280nm}, o-diphenolase activity and NaCl concentration were plotted against fraction number to obtain the elution profile (Fig 2). Fractions 11-19 were pooled and saved for gel filtration chromatography.

Gel filtration chromatography on sephadex G-200

Seven grams of sephadex G-200, (particle size 40-120μ), was soaked in distilled water for 3 days at room temperature to swell. The swollen gel was degassed and packed into a glass column (2.6 x 40cm) at room temperature. The column was then equilibrated at 4°C with 0.1M potassium phosphate buffer of pH 6.8.

To the pooled enzyme fractions from ion-exchange step was added enough solid ammonium sulphate to give 90% saturation. The suspension was immersed in icebath, left for 30 minutes with constant stirring, and then centrifuged at 25,000g for 10 minutes at 5°C. The precipitate was redissolved in 0.1M potassium phosphate buffer of pH 6.8 to give 19ml, which was then applied to the equilibrated sephadex G-200 column. The column was subsequently eluted with the same buffer. A flowrate of 7.5ml per hour was maintained with a peristaltic pump. Three millilitre fractions were collected with a fraction collector. The absorbance of each fraction at 280nm, and o-diphenolase activity were plotted against fraction numbers (Fig.3). Fractions 23-35 were pooled for a second ion-exchange chromatography step. The pooled fraction was concentrated to 8ml against dry sephadex G-100 particles.

Second Ion-exchange Chromatography

A glass column (2.2 x 22cm) was packed with swollen DEAE-sephadex A-50 and equilibrated with 0.1M potassium phosphate buffer as described before. Four millilitres of the concentrated enzyme solution from the gel filtration step was applied to the equilibrated column. Elution was with linear gradient from 0.1M phosphate buffer to 0.25M of same buffer. The flowrate was maintained at 20ml per hour, and 4ml fractions were collected.

Absorbance at 280nm and o-diphenolase activity were determined for each fraction, and plotted against fraction number (Fig.4). Fractions 20-22 were pooled and concentrated against dry sephadex G-100 to 4.0ml. This fraction was referred to as the pure enzyme.

Polyacrylamide-gel electrophoresis

Discontinuous vertical gel electrophoresis was performed according to the method of Davis, (1964). Shandon disc electrophoresis tank was used. The gel was prepared from acrylamide, N,N'-methylene-bisacrylamide (BIS), ammonium persulphate, and N,N,N',N'-tetramethyl ethylenediamine (TENED) in glass gel tubes (0.5 x 7.5cm). Gel concentrations were 7.5% and 2.5% for running and stacking (or spacer) gel respectively. Running gel was prepared in Tris-Hcl buffer pH 9.0, while stacking gel was in Tris-Hcl buffer pH 6.7. Enzyme samples (0.05ml) were applied on top of the stacking gel by mixing with 0.25ml of stacking gel, and a drop of 0.1% bromophenol blue in 50% glycerol. Electrophoresis was performed at a constant current of 5mA per gel at room temperature for 1½ hours, and the electrode buffer was tris-glycinate pH 8.2. After the run, the gels were removed from their supporting tubes. Some of the gels were placed in a solution of 30mM catechol in 0.1M phosphate buffer (pH 6.8) containing 0.05% p-phenylenediamine for 1 hour to detect o-diphenolase activity.

The remaining gels were then placed in 12% trichloroacetic acid solution to fix the proteins and then stained for one hour using 0.25% coomassie brilliant blue in methanol: acetic acid: water (5:1:5 v/v/v). The stained gels were destained in 7% (v/v) acetic acid solution for 12 hours and stored indefinitely in 7% acetic acid. The gels were then photographed (Plate 1) for a permanent record.

Determination of protein content

Protein content of enzyme extracts and pooled fractions were determined according to the method of Lowry et al, (1951) as outlined by Plummer, (1971), using the Folin-Ciocalteau reagent and bovine serum albumin (BSA) as standard.

Protein reacts with the Folin-Ciocalteau reagent to give a coloured complex. The colour so formed is due to the reaction of alkaline copper with the protein, and the reduction of phosphomolybdate by aromatic amino acids particularly tyrosine and tryptophan present in the protein.

A standard curve (Fig. 18) was prepared from which protein concentrations of unknown samples were determined.

Elution of proteins from chromatographic columns was monitored by measuring absorbance at 280nm with a Beckman DB-GT spectrophotometer.

Determination of total carbohydrate content

Total carbohydrate content of enzyme preparations was determined by the Phenol-sulphuric acid method (Dubois et al, 1956).

Simple sugars, oligosaccharides, polysaccharides and derivatives including methyl esters with free reducing groups give an orange-yellow colour with phenol/concentrated H_2SO_4, the intensity of which can be determined spectrophotometrically at 485nm.

Glucose was used as standard. Carbohydrate content of enzyme preparations was determined from a standard plot of absorbance at 485nm versus glucose content (Fig. 19, Appendix III).

Determination of copper content

The copper content of purified enzyme preparations was estimated by atomic absorption spectrophotometry (AOAC, 1975) with a Pye Unicam SP 1900 atomic absorption spectrophotometer.

A solution of cupric nitrate in deionized water containing 1ppm copper (0.038 gm $Cu(NO_3)_2.3H_2O$ in 100ml H_2O diluted 100-fold) served as the standard. Aliquots containing 0.1-1.0 ppm copper of the standard cupric nitrate solution were prepared and their absorbance read at 324.9nm. Copper content of purified enzyme preparations was determined from calibration curve of absorbance at 324.9nm versus copper content (Fig. 20).

Assay of o-diphenolase activity

o-Diphenolase activity was determined spectrophotometrically. The increase in absorbance was measured at wavelengths at which the oxidation products of the different substrates absorb (Table 1). The reference cuvette (1cm light path) contained 2.7ml of 0.1M potassium phosphate buffer pH 6.8 and 0.3ml of 0.3M substrate (to give a final concentration of 30mM in a 3ml reaction mixture) in the same buffer. The sample cuvette contained 2.6ml of the buffer, 0.3ml of the 0.3M substrate in the same buffer, and 0.1ml of the enzyme solution. All solutions were allowed to equilibrate and measurements made at room temperature. Under the above-mentioned conditions, absorbance change was linear during the first 2 minutes, and this was used to calculate the initial rate of enzyme reaction. One unit of enzyme activity was defined as that amount of enzyme that caused a change in absorbance of 0.001 per minute under the described conditions of assay.

Inhibition studies

Several compounds known to reduce the enzymic browning of fruits were tried as inhibitors (Table 6). Conditions for assay were identical as described for enzyme assay except that the sample cuvette contained 2.3ml of 0.1M potassium phosphate buffer pH 6.8, 0.3ml of 10mM inhibitor (to give a final concentration of 1mM in a 3ml reaction mixture) in same buffer, 0.1ml of enzyme solution - incubated for 30 minutes at room temperature - before the addition of 0.3ml of 0.3M catechol in same buffer.

Kinetic Studies

Conditions for assay were the same as for the enzyme assay except that substrate (catechol) concentration was varied (5-50mM) in presence and absence of fixed amounts of selected inhibitors. The kinetic constants Km and Vmax for the crude enzyme preparations from the 10 cultivars of yam (Table 3), Km, Vmax and Ki using various inhibitors for the purified enzyme from D. rotundata cv. Zaria were determined from Lineweaver-Burk plots (Table 7, Figs. 7 and 10) as described by Whitaker, (1972).

Determination of the pH profile of the enzyme

To determine optimum pH, the activity of the crude enzyme preparations from the ten cultivars of yam and the purified enzyme were determined as a function of pH, using catechol as the substrate. The enzymatic assay was carried out as described previously with 0.1M acetate buffer in the pH range of 3.0-6.0, 0.1M phosphate buffer, 6.0 - 8.0, and 0.1M Tris-Hcl buffer for higher pH values.

Effect of pH on Km and Vmax of purified o-diphenolase

The enzyme was assayed as previously described with varying concentrations of catechol at different pHs (Table 4, Appendix IV). The Km and Vmax were determined from Lineweaver-Burk plots prepared with data obtained at each pH value. Relevant plots of these kinetic constants against pH were constructed (Fig. 9) employing the method of Dixon and Webb, (1964).

Substrate specificity

Substrate specificity studies involving the crude enzyme preparations from the ten cultivars of yam and the purified enzyme from D.rotundata cv. Zaria were carried out using several phenolic compounds (mono-, di- and polyphenols). Assay procedures were same as previously described, and readings were taken at the appropriate wavelengths as given in Table 1. The initial rate of oxidation obtained with each of these substrates was expressed as percentage of that obtained with catechol as substrate (Table 5).

Test of the stability of purified o-diphenolase

Solutions of the purified enzyme were left at room temperature, 4°C, and in the frozen state for 14 days. An aliquot of the enzyme was assayed daily for enzyme activity as previously described with catechol (30mM) as substrate.

Thermostability of purified o-diphenolase

Equal amounts of the purified o-diphenolase solution were incubated for 5 minutes at 30°C, 40°C, 50°C, 60°C, 70°C, 80°C, 90° and 100°C in a waterbath, then cooled to room temperature and assayed for activity as previously described using catechol (30mM) as substrate.

Residual activity expressed as percentage of maximum activity was plotted against temperature in ^{o}C (Fig. 11). The heat of inactivation (ΔH) was calculated from the slope of a plot of log. initial velocity versus reciprocal of temperatures (in ^{o}K) (Fig 17), using the Arrhenius equation (Appendix V)

Molecular weight determination

The molecular weight of the purified enzyme was estimated from its elution volume obtained after gel filtration on a calibrated column (2.6 x 40 cm).

Gel filtration is based on fractionation and separation of molecules according to size. Molecules larger than the largest pores of the swollen sephadex, i.e. above the exclusion limit, cannot penetrate the gel particles and therefore pass through the bed in the liquid phase outside the particles. They are thus eluted first. Smaller molecules, however, penetrate the gel particles to a varying extent depending on their size and shape. Molecules are therefore eluted from a sephadex bed in order of decreasing molecular size.

Procedure: A mixture consisting of twenty milligrams of each of the protein standards - Catalase (MW 225,000), D(-) Lactate dehydrogenase (MW 146,000), Bovine serum albumin (BSA) (MW 67,000), Haemoglobin (MW 65,000), egg albumen (MW 45,000), and cytochrome C (MW 12,500) was dissolved in 10mls of 0.1M potassium phosphate buffer (pH 6.8).

Five millilitres of this solution was applied to the column previously equilibrated with the 0.1M phosphate buffer of pH 6.8. The column was eluted with the same buffer, flowrate was maintained at 12ml per hour and 3ml fractions were collected. Elution positions of the marker proteins were determined by the measurement of absorbance at 280nm with a Beckman DB-GT spectrophotometer. Four millilitres of the purified enzyme preparation was then applied to the column. The elution position of the enzyme was ascertained by measurement of o-diphenolase activity of the collected fractions.

Determination of amino acid composition of o-diphenolase

Principle: The working principle of an amino acid analyser is based on an elution chromatography from buffered columns of ion-exchange resin. The separation of the amino acids largely depends on their pKa values and to some extent on the affinity of the side chains for the resin (White et al,1964). The separated amino acids are automatically reacted with ninhydrin reagent and a purple or yellow colour is developed by passage through a coil heated in a waterbath. The absorbance of the resulting effluent is measured with a colorimeter at 570nm for all the amino acids except proline and hydroxyproline which are measured at 440nm and plotted on paper with a strip chart recorder. The area under each peak gives an estimate of the quantity of each amino acid present.

The identification of each amino acid is possible, since, under controlled conditions the position of emergence of each amino acid is constant (the elution time is characteristic of each amino acid).

There are usually two ion-exchange columns - a short column for the analysis of basic amino acids and a long column for the acidic and neutral amino acids.

Preparation of acid hydrolysate

Reagents

1 Constant boiling hydrochloric acid (6N), saturated with nitrogen.

2 Sample buffer (citrate buffer, pH 2.2)

Procedure

Samples for amino acid analysis were hydrolysed with the 6N HCl in sealed ampoules under N_2 at $110^{\circ}C \pm 1^{\circ}$ for 24 hours. Hydrolysis was followed by ion-exchange chromatography in the TSM Technicorn Autoanalyser.

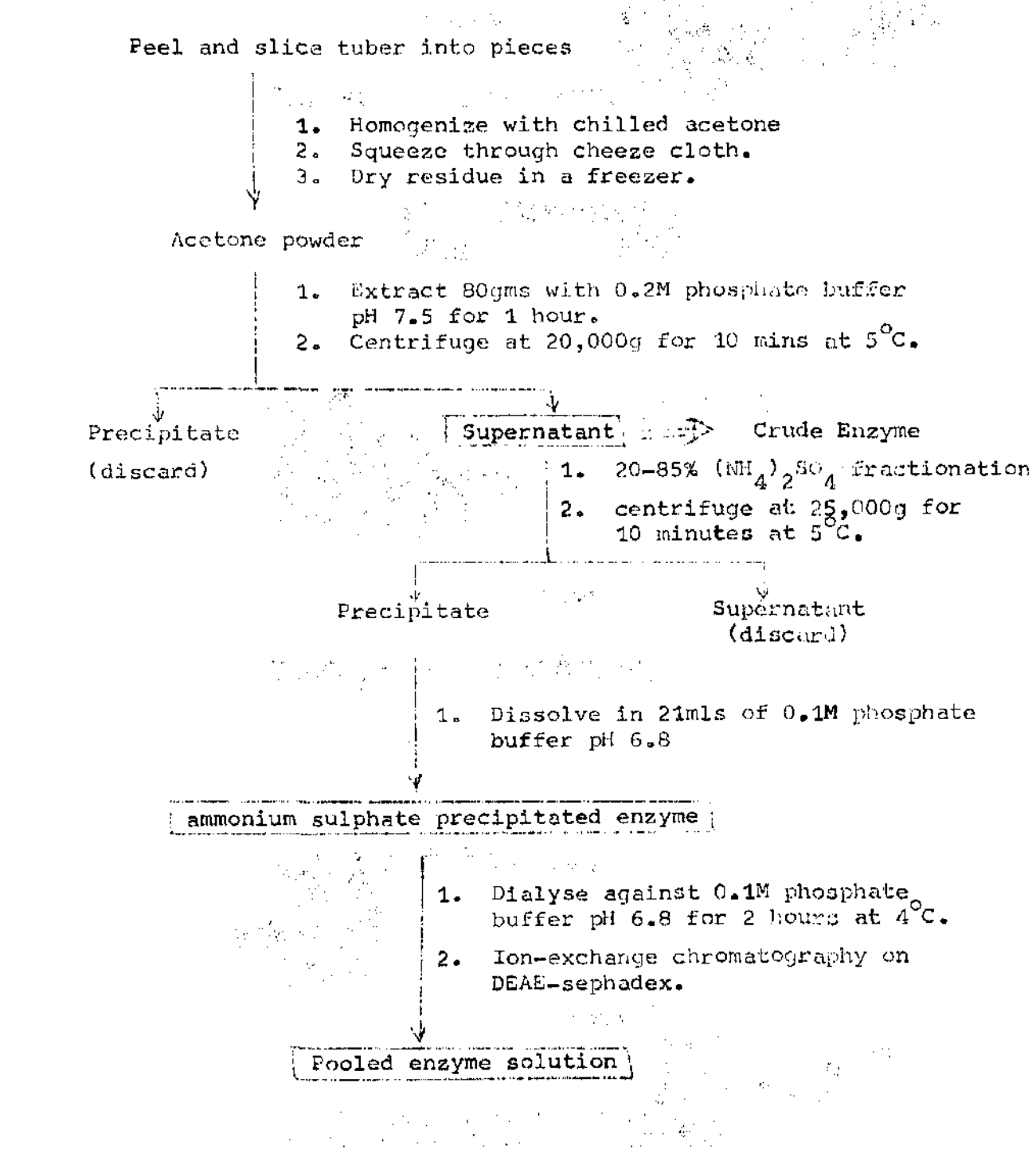
Calibration of the analyser and the estimation of amino acids

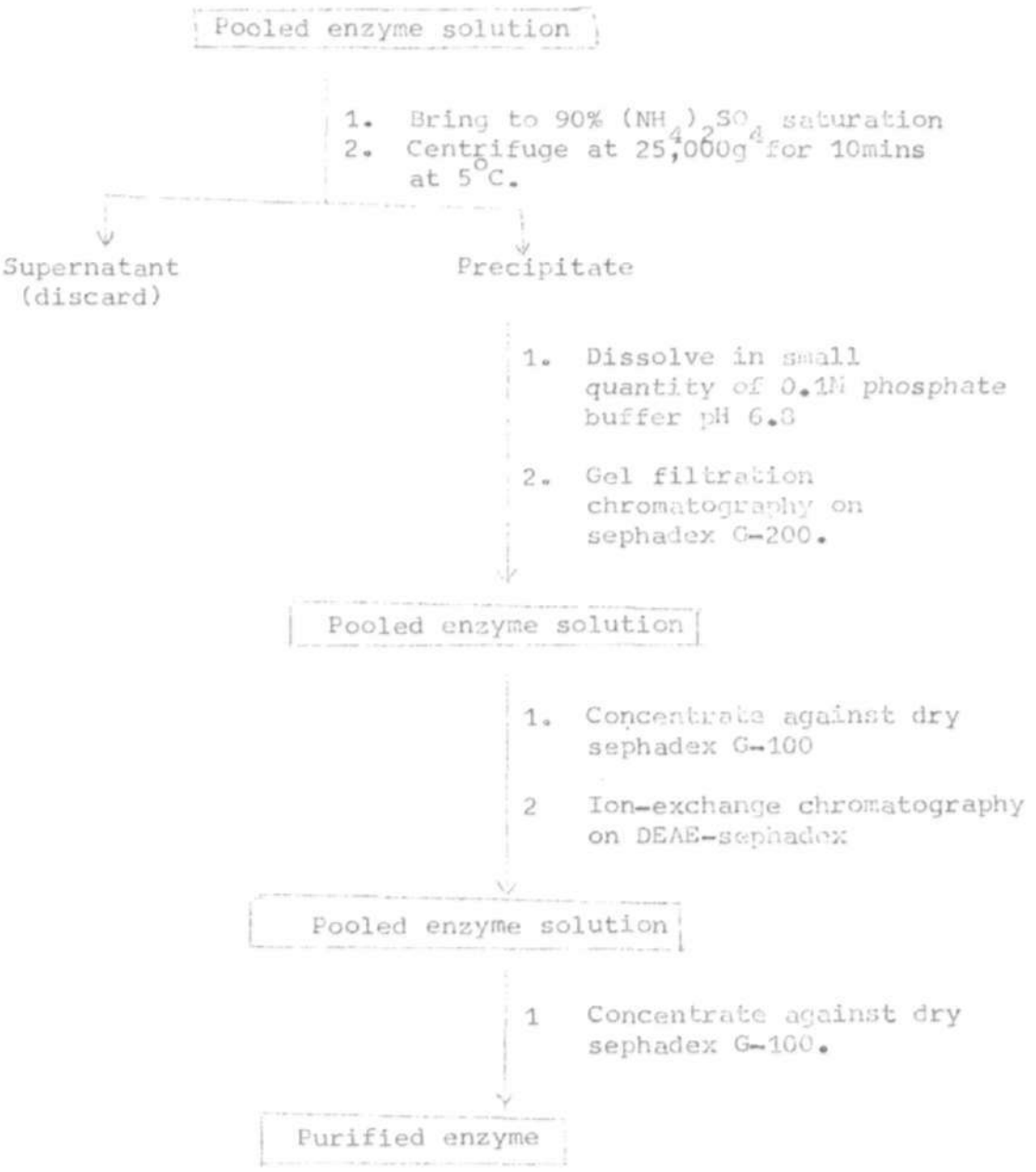
The analyser (Technicon TSM sequential Multisample analyser for amino acids) was calibrated by applying a standard mixture of amino acids (supplied by the Tehnicon Corporation, Tarrytown, N.Y., U.S.A.) containing a known amount (0.25 micromoles) of each amino acid to the column.

The concentration of each amino acid present was indicated by the integrated area under the peak representing it on the chromatogram. The area of each peak was integrated in terms of its absorption values and multiplied by its width in terms of the plotted points at half peak height.

The concentration of each amino acid present in the hydrolysate was determined by comparing the integrated area under its peak with that of the corresponding standard with known concentration.

Flow-diagram for the purification of o-diphenolase from white yam (Dioscorea rotundata cv. Zaria) tuber




Pooled enzyme solution
1. Bring to 90% $(NH_4)_2SO_4$ saturation
2. Centrifuge at 25,000g for 10mins at 5°C.
Supernatant (discard)
Precipitate
1. Dissolve in small quantity of 0.1M phosphate buffer pH 6.8
2. Gel filtration chromatography on sephadex G-200.
Pooled enzyme solution
1. Concentrate against dry sephadex G-100
2 Ion-exchange chromatography on DEAE-sephadex
Pooled enzyme solution
1 Concentrate against dry sephadex G-100.
Purified enzyme

Absorption spectra of oxidized substrates

Determination of the absorption spectra of oxidized substrates resulted in the absorption maxima listed in Table 1. Based on these, a wavelength was selected at which the enzyme was assayed with each substrate.

Table 1 - Wavelengths at which the spectrophotometric determination of o-diphenolase activity on various substrates was carried out

Substrate	wavelength (nm)	
	this study	other studies
Catechol	390	400 (Benjamin and Montgomery, 1973)
Chlorogenic acid	400	400 "
		440 (Interesse et al, 1980)
3,4 di-hydroxy phenylalamine (Dopa)	475	475 "
		460 (Benjamin and Montgomery, 1973)
Pyrogallol	320	334 "
3,4,5 trihydroxybenzoic acid	395	-
p-cresol	400	400 (Benjamin and Montgomery, 1973)
Quinol	400	400 (Chaplin, 1978)
L-tyrosine	472	472 (Benjamin and Montgomery, 1973)
p-coumaric acid	410	410 (Interesse et al, 1980)

It should be noted that the absorption spectra of the oxidation products do not reveal discrete maxima, but only shoulders. The values selected were median points of the shoulders.

Extraction and purification of o-diphenolase

To test the degree of purification of the enzyme after each purification step, the volume, total protein and enzymatic activity of the various enzyme fractions were measured. o-Diphenolase activity was monitored using 30mM catechol in 0.1M phosphate buffer of pH 6.8 as substrate. Table 2. shows the results obtained.

Table 2 - Purification profile of D.rotundata cv. Zaria o-diphenolase

Purification step	Volume (ml)	Protein (mg)		o-diphenolase activity (units)		Specific activity^{-1} units.mg	Recovery or Yield %	Purification fold
		mg ml^{-1}	total	units.ml^{-1}	Total			
1 Crude extract from acetone powder	190	17.30	3286.43	1400	266000	80.94	100.00	1.00
2 20-85% ammonium sulphate fractionation and analysis	34	74.39	2529.40	7800	265000	104.85	99.70	1.30
3 Chromatography on DEAE-Sephadex	95	9.68	919.13	1350	128250	139.54	48.21	1.72
4 Gel-filtration Sephadex G-200	30	0.50	15.00	4030	120900	8060.00	45.45	99.58
5 Second chromatography on DEAE-sephadex	12	0.20	2.42	2000	24000	9901.00	9.02	122.33

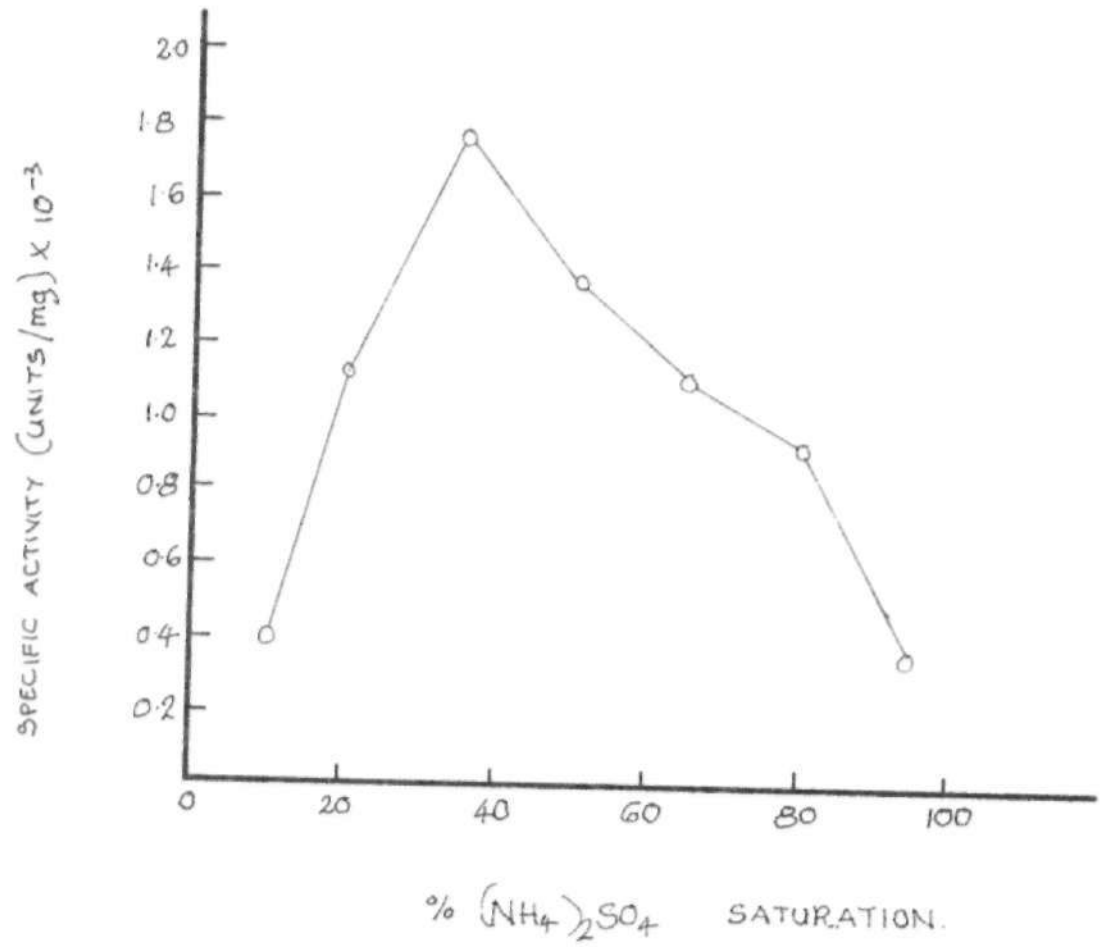


FIG. 1. - Ammonium sulphate fractionation of crude o-diphenolase.

Ammonium sulphate fractionation

Figure 1 shows the result of $(NH_4)_2SO_4$ fractionation test carried out on crude enzyme extract from acetone powder, to determine the best fractionation range to use in the purification. Table 2 shows that a 20-85% $(NH_4)_2SO_4$ cut did not increase the fold purification of the crude enzyme by much.

Ion-exchange chromatography

Figure 2 shows the elution profile of the protein and the activity of o-diphenolase on catechol obtained in ion-exchange chromatographic run (DEAE-sephadex column (2.6 x 40cm))of the dialysed ammonium sulphate precipitated enzyme.

The chromatographic result shows that it is possible to isolate an enzymatically active fraction which represents a large part of the applied protein. The data on Table 2 show that the purification fold of the enzyme was only slightly increased by ion-exchange chromatography.

Gel-filtration chromatography

The elution profile of o-diphenolase from sephadex G-200 column revealed broad improperly resolved peaks (Fig.3). Data on Table 2 show that gel filtration chromatography of the enzyme from DEAE column resulted in almost 100-fold purification over the starting material.

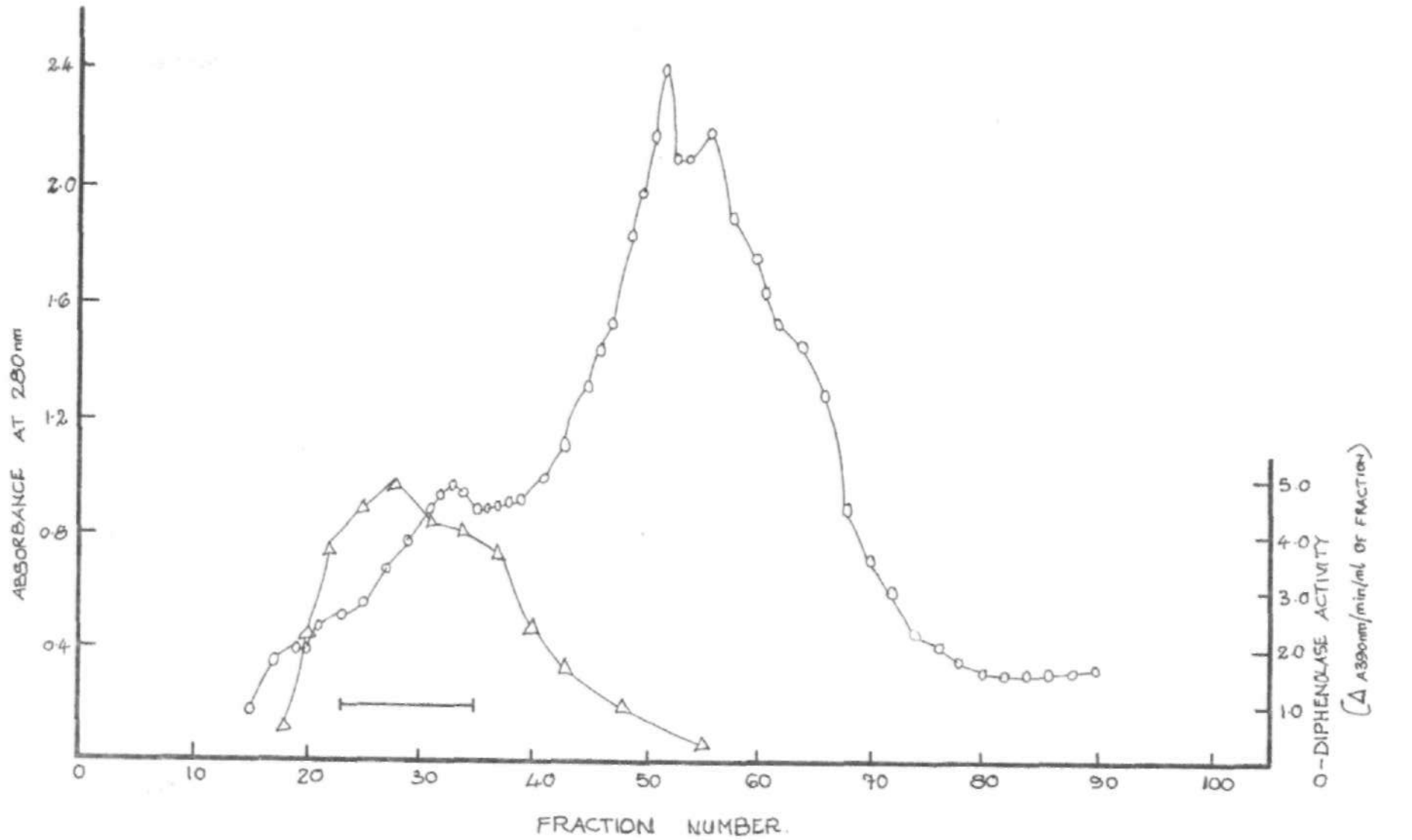


FIG. 3.- The elution profile of enzyme fraction from the ion-exchange chromatography step on Sephadex G-200 column (2.6 x 40cm). Elution was with 0.1M Phosphate buffer pH 6.8. The flowrate was 7.5ml/hour at 4°C and 3ml fractions were collected. O-O-O absorbance at 280nm; Δ-Δ-Δ, o-DPO activity (ΔA_{390}nm/min/ml). Fractions pooled as indicated ⊢——⊣

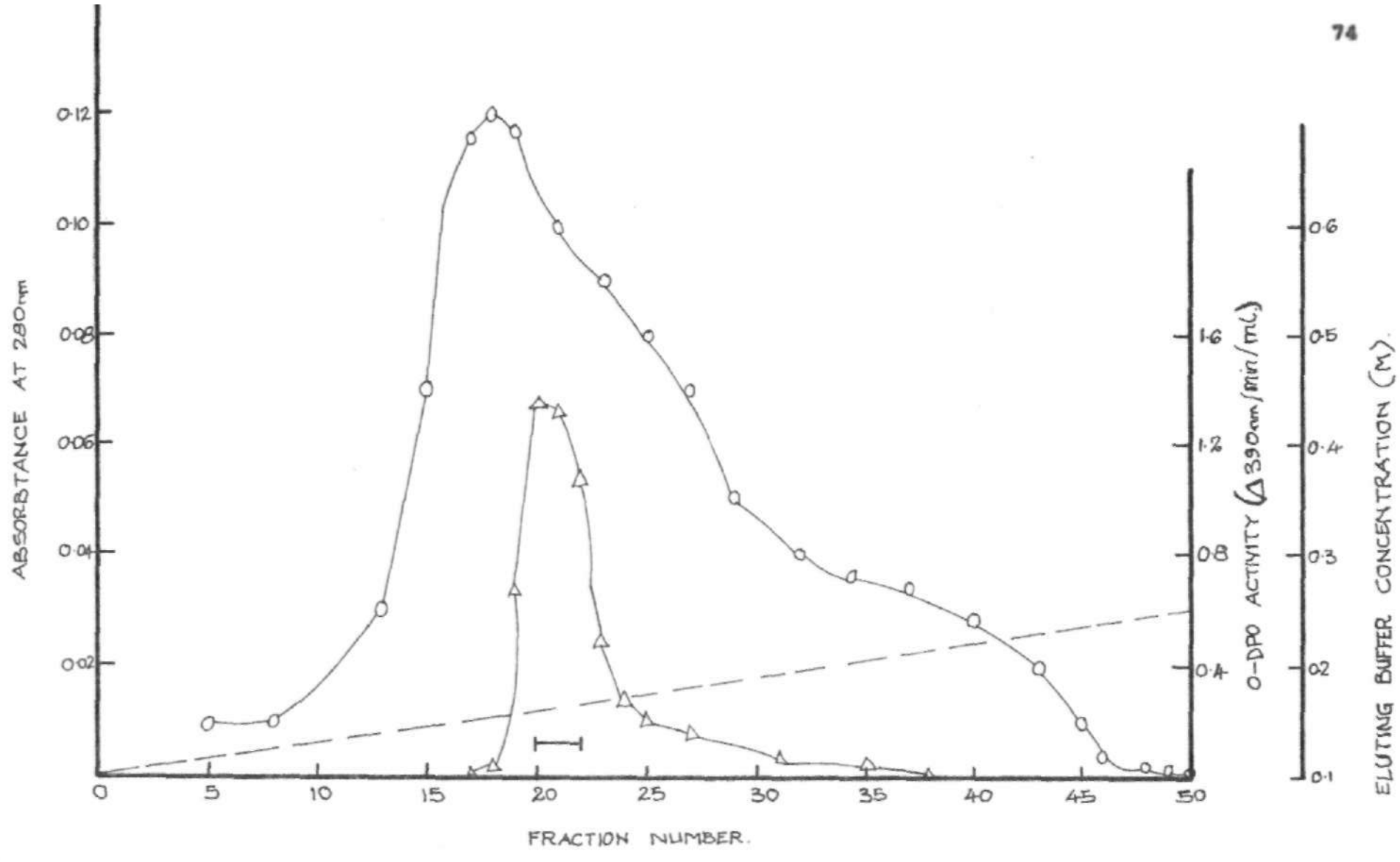


FIG. 4.- Chromatography of the enzyme fraction from sephadex G-200 column on a DEAE-sephadex column (2.2 x 22cm), eluted with a linear gradient from 0.1M to 0.25M Phosphate buffer pH 6.8. Flowrate was 30mls/hour, 4ml fractions collected. O–O–O absorbance at 280nm; Δ–Δ–Δ, o–DPO activity (ΔA_{390}nm/min/ml); - - -, concentration of eluting buffer.

On subsequent passage of the enzyme from the gel filtration step through a second column (2.2 x 22 cm) of DEAE-sephadex (Fig.4), a 122-fold purification over the crude enzyme extract was accomplished. A high value of specific activity (9901 units mg^{-1}) was also obtained after this final purification step.

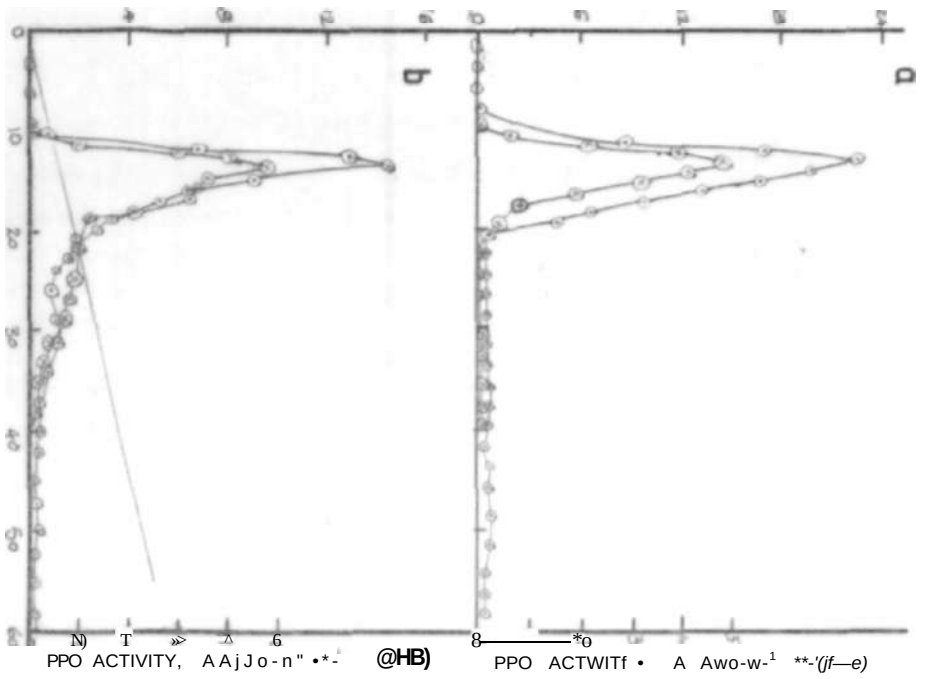
OBSERVATIONS ON PURIFICATION PROCEDURE

Attempts to purify the dialysed ammonium sulphate precipitated enzyme by gel filtration failed because the enzyme solution was too viscous and slimy (mainly due to presence of glycoproteins), and did not enter the sephadex G-200 column at 4°C. For more than 5 hours after sample application, the sample was still on top of the gel. Dilution of the sample did not effect any change. When tried at room temperature, there was little movement of the sample into the column, but the extract appeared to cause hydrolysis or dissolution of the sephadex gel material.

It therefore became somewhat compulsory to carry out ion-exchange chromatography first. This resulted in little or no increase in purification fold (Table 2), but gave a less slimy enzyme preparation which then passed through sephadex G-200 column and resulted in almost a 100 fold purification over the starting material.

Elution profiles of o-diphenolase in different chromatographic runs of the ammonium sulphate fraction on DEAE-sephadex column (2.6 x 40cm) under different eluting conditions. Elution was with (a) 0.1M phosphate buffer pH 6.8 (b) linear gradient from 0.1M Tris-Cl buffer pH 8.3 to 0.5M NaCl in same buffer (c) 0.2M Tris-HCl buffer pH 8. (d) linear gradient from 0.1M Tris-HCl buffer pH 9 to 0.5M NaCl in same buffer.

ABSOR&ANCE AT *ZGOnm*



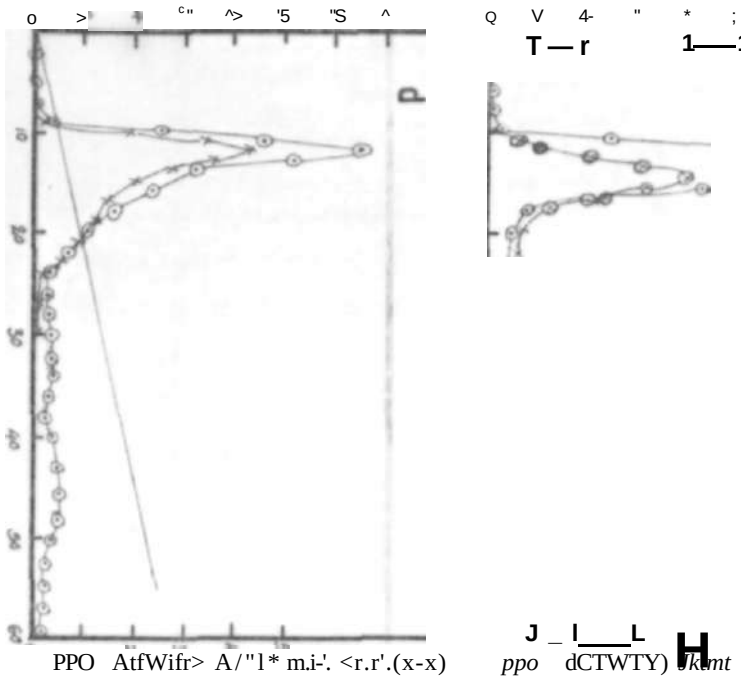
PPO ACTIVITY, AAjJo-n" •*- @HB)

PPO ACTWITf • A Awo-w-[1] **-'(jf—e)

ftODIlitf* CHLORIC? COWC. C H)

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A650f»«ANCC AT *ZaOrm* C°~°)



PPO AtfWifr> A/"l* m.i-'. <r.r'.(x-x)

ppo dCTWTY) *Jkimt* wfcH.w* 0 s - > 0

•>OD*UM cnwd!jt COMC. CM)'

Also, passage of the concentrated enzyme from the gel filtration step through an equilibrated (with 0.1M phosphate buffer of pH 6.8) calcium phosphate gel-cellulose (prepared according to the method of Massey, (1960) as reported by Koike and Hamada, (1971) column did not increase purification fold. So the method was abandoned as a means of purifying D. rotundata cv. Zaria o-diphenolase.

Effect of column conditions on the elution of o-diphenolase from DEAE-sephadex column

Figures 2a, b, c and d show the elution profiles of the protein and o-diphenolase activity on catechol in different chromatographic runs of the dialysed ammonium sulphate precipitated enzyme on DEAE-sephadex column (2.6 x 40 cm) under different eluting conditions. The elution position of the o-diphenolase virtually remained the same despite the changing eluting conditions (see Figs 2a, b, c, and d). This shows that the enzyme's conformation may be such that there are only few ionizable groups on the outside of the enzyme molecule.

Disc gel electrophoresis

The results of the electrophoresis runs are shown in Plate 1. The ammonium sulphate precipitated enzyme gave several protein bands when stained with coomassie brilliant blue (Disc A). The pooled enzyme solution after the first ion-exchange chromatography on DEAE-sephadex revealed two protein bands (Disc B). The purified enzyme revealed a single protein band (Disc C).

On incubation with catechol in 0.1M phosphate buffer of pH 6.8, the o-diphenolase activity could be detected only in the protein corresponding to the stained band (Disc. D). Disc E had no sample in it.

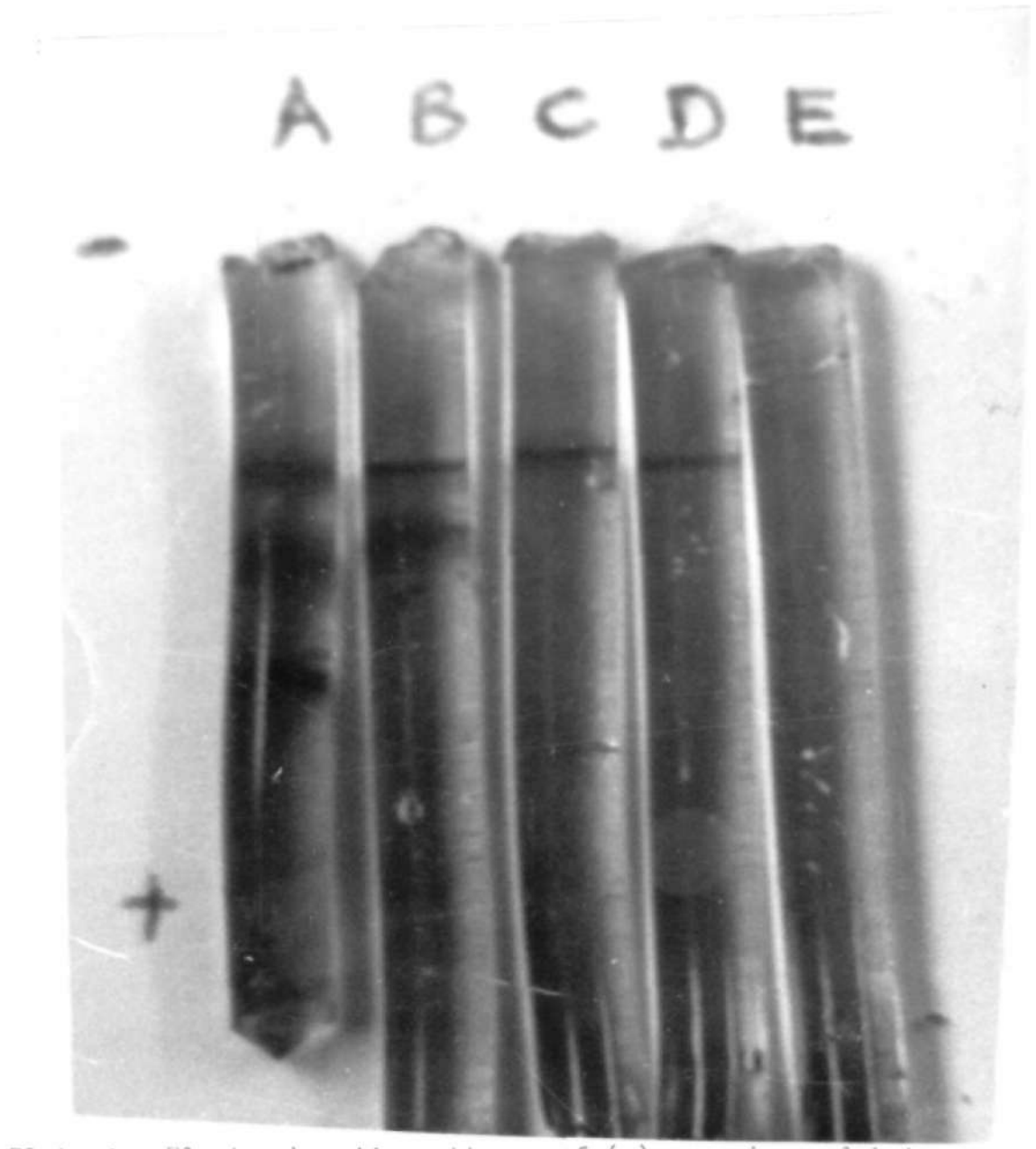


Plate 1 - Electrophoretic patterns of (a) ammonium sulphate precipitated enzyme; (b) pooled enzyme solution after first ion exchange chromatography; (c) purified enzyme preparation stained with coomassie brilliant blue; (d) purified enzyme preparation band detected with catechol; (e) bromophenol blue only.

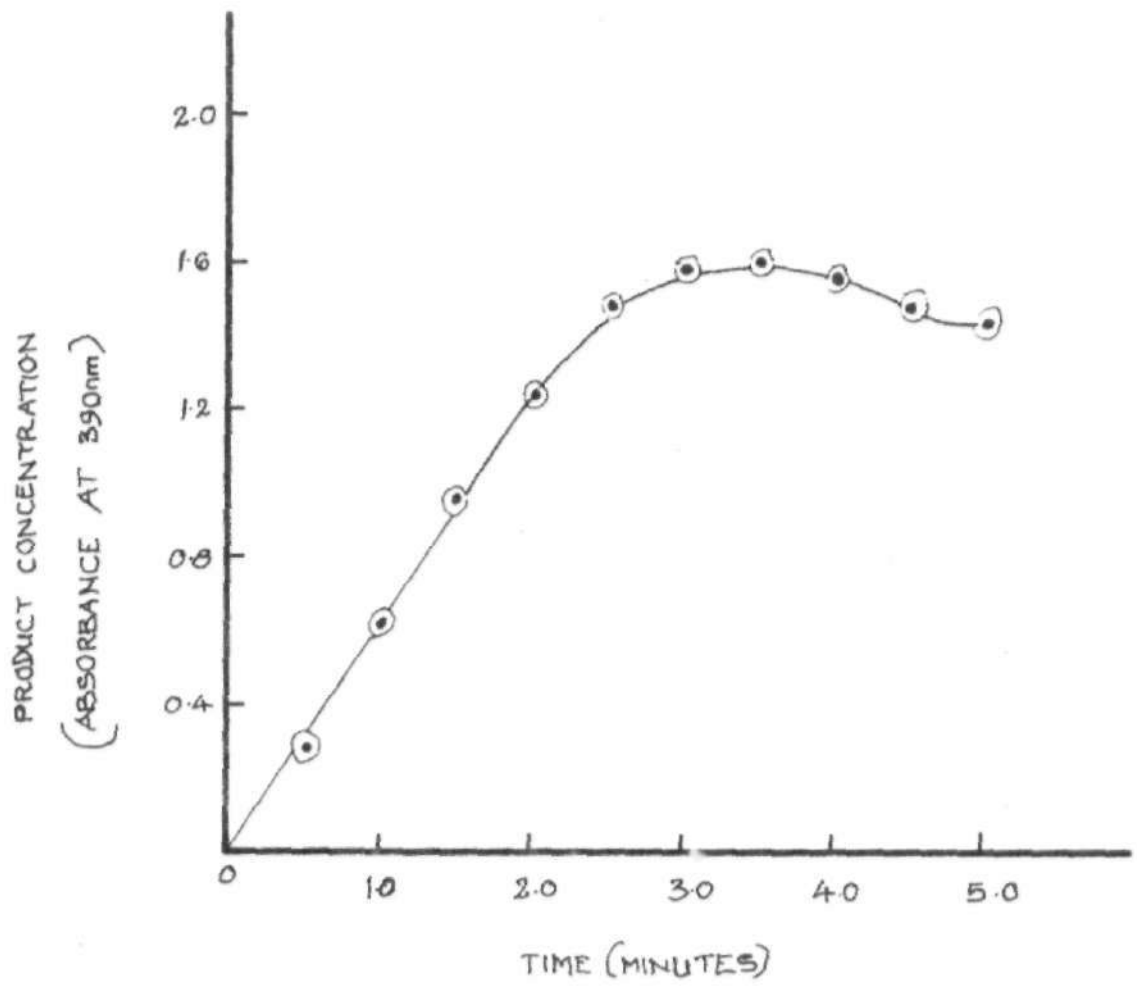


FIG. 5.- Plot of absorbance at 390nm of product formed versus time representing rate of oxidation of catechol (30mM) by o-diphenolase at pH 6.8. Absorbance change was linear for about two minutes.

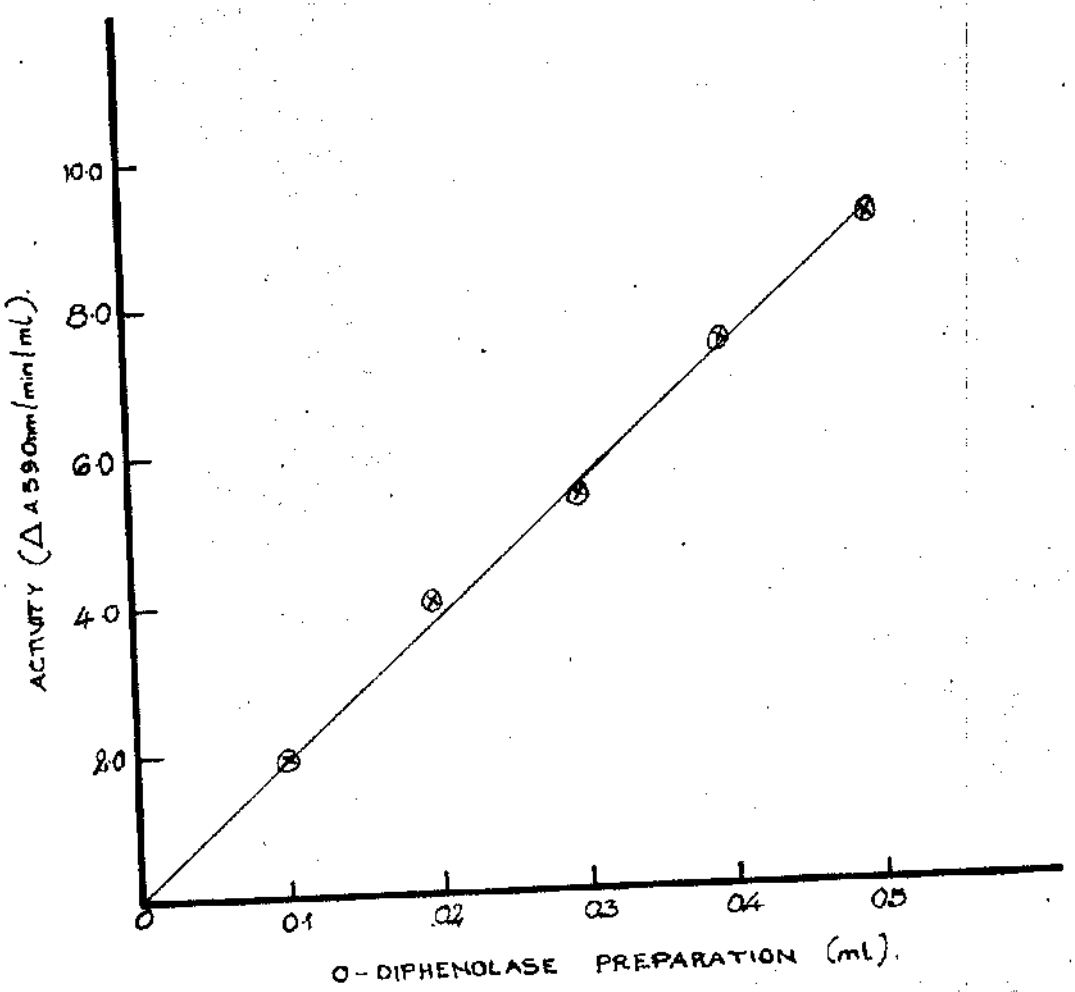


FIG. 6.- Plot of initial rate of oxidation of catechol (30mM) versus the concentration of purified o-diphenolase

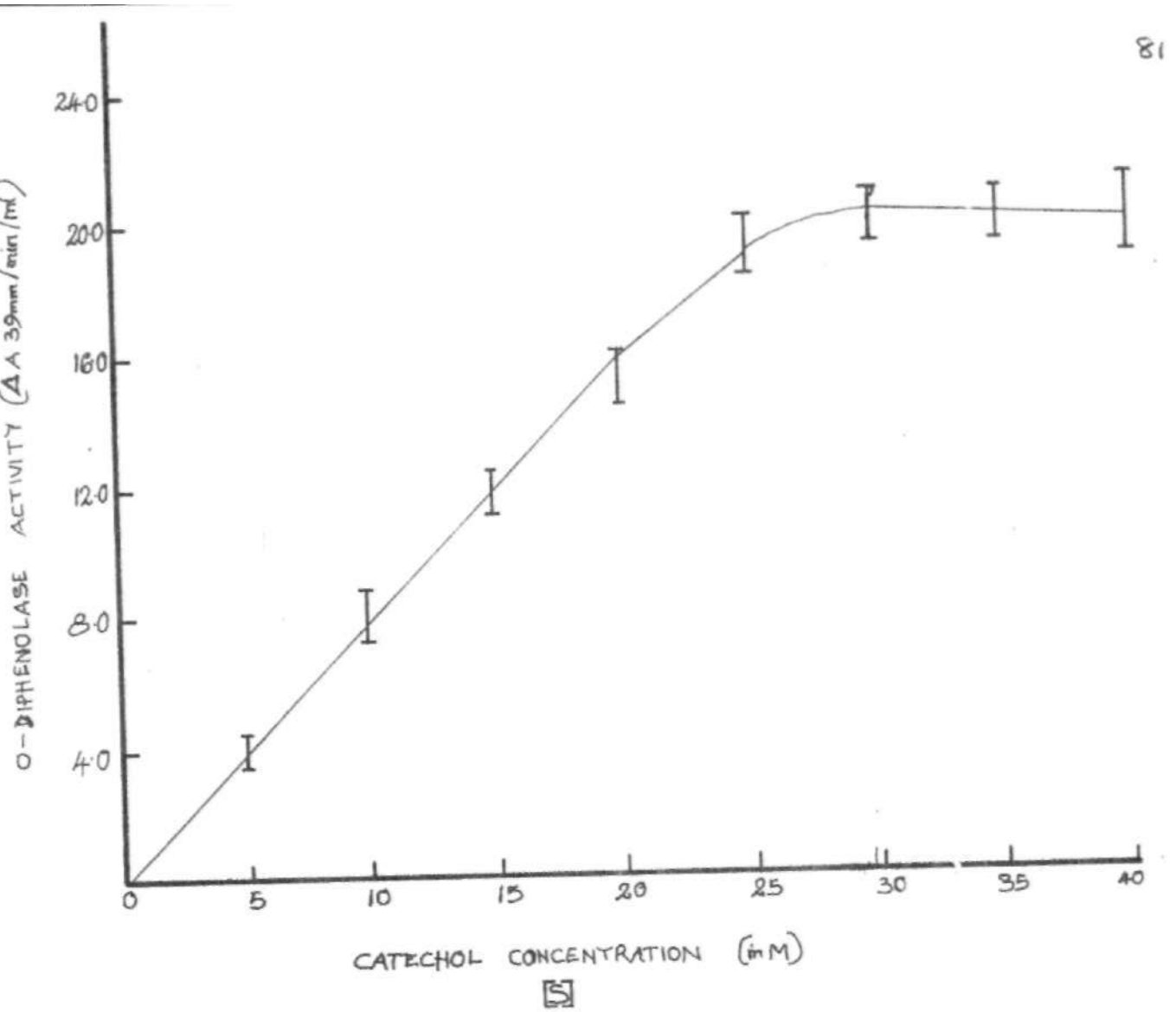


FIG. 7. - Plot of o-diphenolase activity (ΔA_{390} nm/min/ml) versus substrate concentration (mM) catechol). Points are shown with their standard deviations from the mean of 4 different determinations). The pH of assay medium was 6.8

Enzyme assay

Under the conditions of assay outlined in the experimental section, o-diphenolase activity on catechol was found to be linear for 2 minutes (Fig.5). Also, activity increased linearly with increasing enzyme concentration (Fig. 6), and with increasing substrate (catechol) concentration (Fig. 7).

o-Diphenolase content of yam cultivars

The amount of o-diphenolase activity in the ten cultivars (expressed in units per gm fresh tuber weight) were estimated using catechol (30mM) as substrate (see Appendix II). Table 3 shows that D.bulbifera cv. Adu had the highest amount of o-diphenolase per unit of tuber weight, while the D. alata cultivars had the lowest.

Kinetic parameters of o-diphenolases from ten cultivars of yam

The reaction of o-diphenolase with catechol was found to follow Michaelis kinetics. The Michaelis constant for the enzyme-substrate complex (Km) and the maximum velocity (Vmax) of o-diphenolases from the ten cultivars of yam were determined from double-reciprocal plots of Lineweaver and Burk, as described by Whitaker, (1972) with catechol as substrate. Data on Table 3 show that the enzyme from D. alata cv. Igiehua had the highest Km value, while D. bulbifera cv. Adu enzyme had the lowest Km value. The Km value of o-diphenolase from D.rotundata cv. Zaria decreased from 13.33mM to 12.50mM on purification. Cultivar Adu o-diphenolase had the highest Vmax, while cv. Igiehua had the lowest.

Table 3 - Some properties of o-diphenolases from different yam cultivars

Cultivar (o-diphenolase source)	o-Diphenolase (catecholase) activity per gm. fresh tuber weight (units) x 10^{-3}	Relati e o-diphenolase activity	Optimum pH	Km (mM)	Relative Vmax (ΔA_{390} nm/min/ml)
D.rotundata					
1 Zaria	22.1450	1.000	5.5-7.0	13.33	1.00
2 Amara	26.8550	1.213	4.5,6.8-7.2	14.28	1.20
3 Orhohozar	9.0867	0.410	4.5,5.5-7.0	11.11	0.60
4 Omi	31.6360	1.429	4.6,6.5-7.2	10.00	1.84
5 Obiaoturugo	37.6040	1.698	5.5-7.2	10.00	2.00
D. alata					
6 Igiehua	4.9459	0.223	5.0,6.8	16.60	0.19
7 Mbala	4.8032	0.217	4.5-5.0,6.5-7.0	14.28	0.21
D. cayenensis					
8 Oku	19.2210	0.868	6.0-7.0	9.09	1.12
9 Ikpen	8.1146	0.366	5.5-7.2	14.28	0.39
D. bulbifera					
10 Adu	41.0660	1.854	5-6, 7.2	8.33	3.52

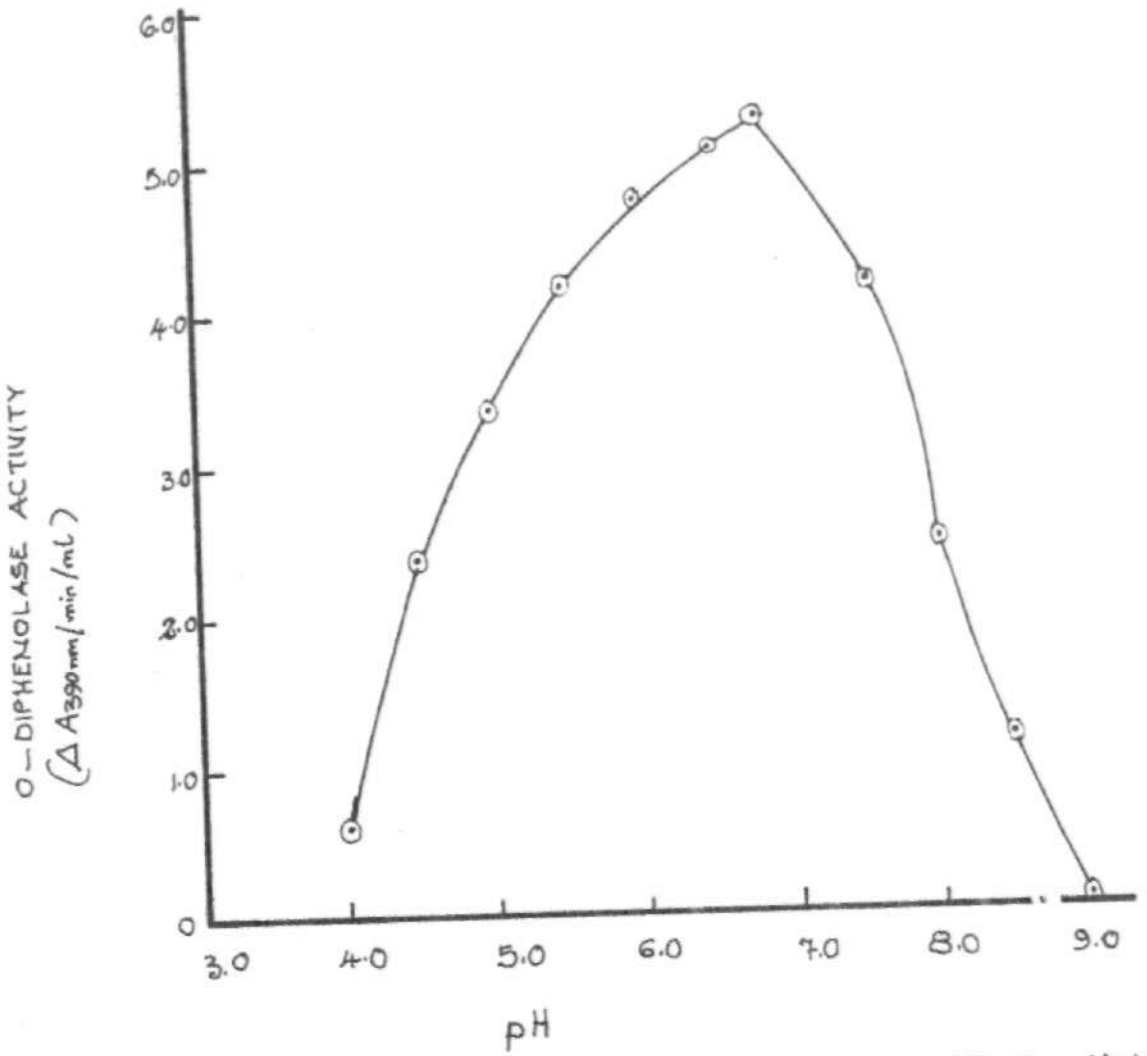


FIG. 8. - Effect of pH on activity of purified o-diphenolase

Effect of pH

pH optima

The effect of pH on the activity of crude o-diphenolases from the ten cultivars of yam used was studied using catechol (30mM) as substrate. Data on Table 3 show that all the cultivars' enzymes have pH optima around neutrality. Some had other pH optima in addition.

Figure 8 illustrates pH activity profile of purified o-diphenolase from D. rotundata cv. Zaria. Maximum activity occurred at pH 6.8. The pH curve is characterized by a rapid decrease in enzyme activity at alkaline pH values, and at acidic pH values below pH 5.5.

Effect of pH on Km and Vmax

Activity of purified o-diphenolase was determined at different substrate (catechol) concentrations and at several pH values over the pH range 4-9, and the data analyzed by Lineweaver - Burk plots (see Appendix IV, Table 10 and Table 4).

Table 4 - Effect of pH on Km and Vmax of purified o-diphenolase with catechol as substrate

pH	Km(mM)	Vmax (ΔA_{390}nm/min/ml)
4.0	90.91	6.67
5.0	19.23	15.87
6.0	12.50	15.87
6.8	12.50	20.00
8.0	55.60	3.63

Figure 9 shows the effect of pH on Vmax, $\frac{Vmax}{Km}$ and Km plotted by the method of Dixon and Webb, (1964). From the plots, groups with pK values around 4.7 and 7.2 were identified to be involved in the catalytic activity of purified o-diphenolase on catechol as substrate and the preservation of native conformation of the active site.

Substrate specificity

Various monophenolic, o-diphenolic and polyphenolic compounds were used to determine the substrate specificities of the crude enzymes from the ten cultivars of yam and of the purified cv. Zaria enzyme. The rate of the reaction for the different substrates was measured at the wavelengths given in Table 1. The results of these assays, expressed as percentage of activity towards catechol (Table 5) show that the o-diphenolases from all the cultivars were active towards all the o-diphenols tested but had little or no activity towards monophenols. Of all the substrates tested, catechol was found to be the most active as a substrate for o-diphenolase, followed by 3,4-dihydroxy phenylalanine (dopa). o-Diphenolases of cultivars Zaria and Adu were slightly more active towards chlorogenic acid than dopa.

It was on the basis of these observations that this enzyme was called o-diphenolase instead of polyphenol oxidase or simply phenolase.

Table 5 - Relative substrate specificities of o-diphenolases from ten cultivars of four species of yam grown for food in Nigeria

Substrate	Activity relative to catechol as substrate										
	cv. Zaria o-diphenolase		Amara	Orhohozar	Omi	Obiatu-rugo	Igiehua	Mbala	Oku	Ikpen	Adu
	crude	purified									
Catechol	100	100	100	100	100	100	100	100	100	100	100
3,4-dihydroxy-phenyl alanine	54.2	68.2	61.5	56.3	54.1	66.7	47.5	42.0	63.9	58.3	48.5
Chlorogenic acid	58.3	80.3	51.3	25.0	33.3	44.4	38.3	32.0	33.3	33.3	50.0
Pyrogallol	9.4	12.8	7.7	12.5	7.4	11.1	3.5	2.1	16.7	12.5	7.7
3,4,5-trihydroxy benzoic acid	0.5	0	0.2	0.6	1.9	2.02	1.1	1.2	0.9	1.3	0.05
p-cresol	.01	0	0.01	0.02	0.14	0.10	0.0	0.0	0.01	0.01	0.01
L-Tyrosine	0	0	0	0	0	0	0	0	0	0	0
Quinol	0	0	0	0.01	0.01	0.01	0	0	0	0	0
p-Coumaric acid	0	0	0	0	0	0	0	0	0	0	0

Concentration of substrate used was 30mM for all.

Inhibition studies

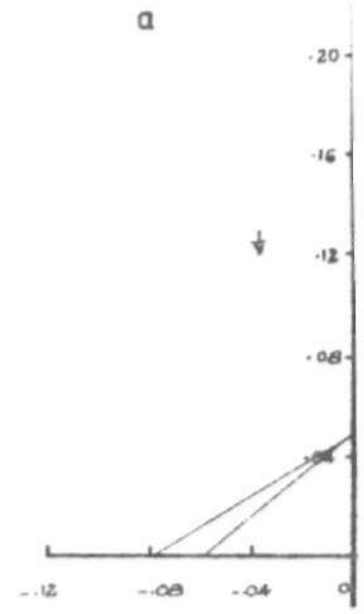
The results of the effects of 1mM concentration of various inhibitors on the crude and purified enzyme preparations using catechol (30mM) as substrate are shown in Table 6.

Cysteine, dithiothreitol, diethyldithio carbamate, sodium metabisulphite and ascorbic acid inhibited the pure enzyme completely. EDTA showed no inhibition of both crude and purified enzyme. The inhibition by potassium cyanide, citric acid, sodium benzoate and sodium azide was greater on the purified enzyme than on the crude preparation. Sodium chloride (even at 1M concentration) had no inhibitory effect on the crude or purified o-diphenolase.

Table 6 - The effect of various inhibitors (1mM) on o-diphenolase activity

Inhibitor	Inhibition (%)*	
	Crude extract	Purified enzyme
Control	0	0
N-acetylimidazole	5.6	8.1
Dithiothreitol (Cleland's reagent)	100	100
Diethyldithiocarbamate (DEDTC)	100	100
Iodoacetic acid	10.0	16.2
Iodosobenzoic acid	79.1	78.4
p-Hydroxybenzoic acid	30.2	40.5
L-Ascorbic acid	91.9	100
Potassium cyanide	80.6	95.8
Malcic hydrazide	4.3	4.8
Ethylenediamine tetraacetic acid (EDTA)	0	0
Citric acid	3.2	11.8
Sodium metabisulphite	100	100
Sodium sulphite	5.2	17.7
Sodium benzoate	15.2	19.1
p-Chloromercuric benzoate	19.6	22.1
2-Mercaptoethanol	99.1	99.6
Sodium azide	10.1	14.3
L-Cysteine	100	100
NaCl	0	0

* The enzyme was preincubated with the inhibitors at room temperature for 30 minutes.



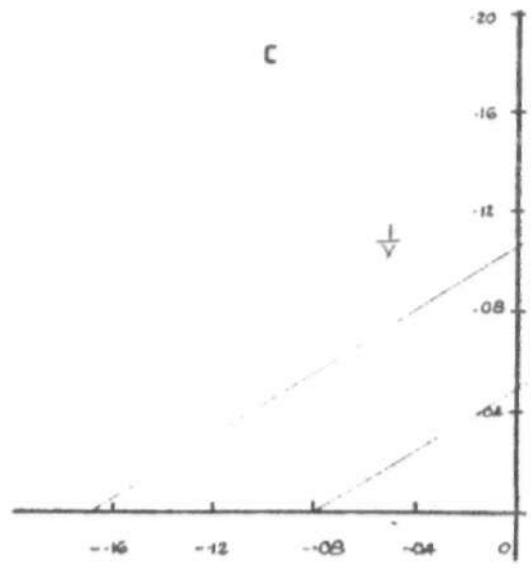


FIG. 10 -

Lineweaver -Burk p
mercaptoethanol (a
0.1M Phosphate buf

The effects of 2-mercaptoethanol, cysteine, sodium azide and potassium cyanide on Km and Vmax of purified enzyme were determined by the double reciprocal method of Lineweaver-Burk (Appendix IV , Fig. 10). The results obtained are shown in Table 7.

Table 7 - Effect of selected inhibitors on the Kinetic constants of purified o-diphenolase

Inhibitor	Km (mM)	Apparent Km (mM)	Vmax (ΔA_{390} nm/min/ml)	Apparent Vmax (ΔA_{390} nm/min/ml)	Type of inhibition	Ki (mM)
Control	12.5	-	20.0	-	-	-
2-mercapto-ethanol (0.167mM)	-	16.7	-	20.0	compe-titive	0.502
Cysteine (0.1mM)	-	25.0	-	20.0	"	0.100
Cysteine (0.167mM)	-	33.0	-	20.0	"	0.100
NaN_3 (2mM)	-	5.9	-	9.4	uncom-petitive	1.776
KcN (1mM)	-	71.4	-	20.0	compe-titive	0.210
KcN (0.667mM)	-	52.63	-	20.0	"	0.210

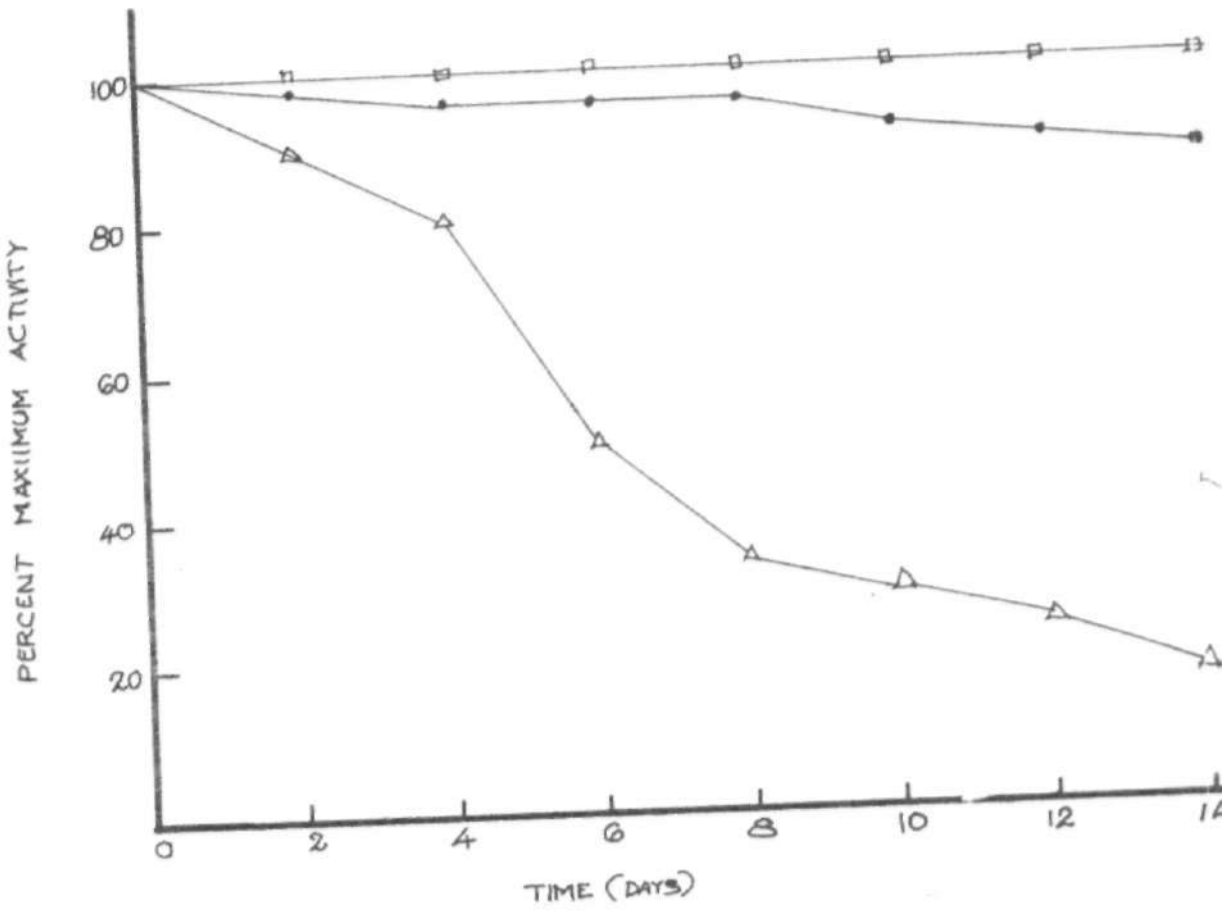


FIG. 11:- Plot of percent maximum activity versus Time (days) showing stability of o-diphenolase preparation at room temperature (Δ - Δ - Δ), 4°C (●—●—●) and in the frozen state (□—□—□). (100% corresponds to ΔA_{390} nm/min/ml = 16)

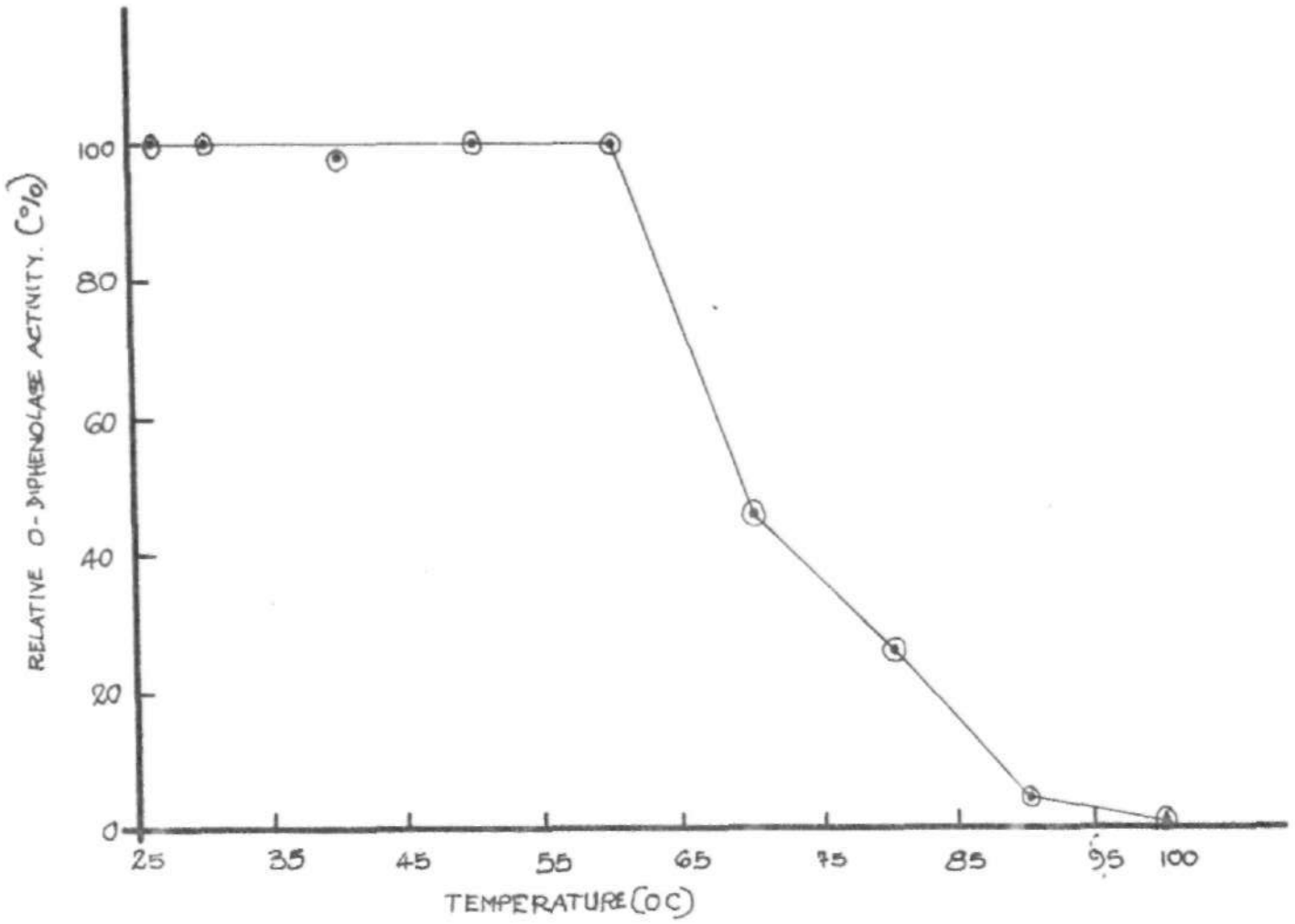


FIG. 12. - Effect of heat on the activity of o-diphenolase. Enzyme preparations were exposed to the various temperatures for 5 minutes, cooled and then assayed at room temperature, in 0.1M phosphate buffer pH 6.8 with 30mM catechol.

Protein and carbohydrate content of purified o-diphenolase

Four millilitres of the purified o-diphenolase was extensively dialysed against frequent changes of distilled water at 4°C for 12 hours to remove the buffer salts, and then lyophilised to dryness with Edwards Modulyo freeze dryer. The dry active o-diphenolase was found to be 75.6% protein and 24.2% carbohydrate (see Appendix III).

Attempts to determine the nature of the carbohydrate component were not successful.

Stability of purified o-diphenolase

Figure 11 shows the stability of purified o-diphenolase on storage for 14 days at room temperature, 4°C and in the frozen state. The plot of relative activity (as % of initial activity) versus time (days) (Fig. 11) shows that at room temperature, the activity of o-diphenolase decreases rapidly and that the enzyme is stable when stored in the frozen state.

Effect of heat and heat inactivation

Figure 12 shows the effect of heating at various temperatures for 5 minutes on the activity of purified o-diphenolase. Exposure of the enzyme to temperatures up to 60°C for 5 minutes did not alter the activity of o-diphenolase. There was a sharp drop in activity on exposure of the enzyme to heat at 70°C for 5 minutes.

Heat of inactivation of o-diphenolase (ΔH) estimated from Arrhenius plot was found to be 26.69Kcal. (see Appendix V, Fig. 17).

UV spectrum of purified o-diphenolase

o-Diphenolase in 0.1M phosphate buffer of pH 6.8 exhibited a typical protein spectrum (high absorbance around 215nm with an additional peak at 280nm) (Fig. 13). There was no significant absorption above 300nm. A solution containing 1.0mg of purified o-diphenolase protein per ml had an absorbance of 1.42 at 280nm and 1.26 at 260nm. The ratio of the absorbance at 280nm and 260nm was found to be 1.13.

Infra-red (IR) spectrum of purified o-diphenolase

Figures 14a and b illustrate the IR spectra for ammonium sulphate precipitated enzyme and purified o-diphenolase respectively. The IR spectrum of purified o-diphenolase showed a broad absorption around 3400-3300cm^{-1} (attributed to -OH groups), intense absorption around 1650cm^{-1} (due to stretching vibrations of C= O groups) and additional absorptions around 1080cm^{-1} and 2100cm^{-1}.

Molecular weight determination

The elution positions of the marker proteins and the purified o-diphenolase activity on a calibrated column of sephadex G-200 is shown in Fig. 15. The molecular weight of purified o-diphenolase was estimated from the plot of elution volume (Ve) in mls against logarithm of molecular weights of marker proteins (Fig.16). Mean molecular weight of o-diphenolase from 3 different runs was estimated to be 107, 333 $\pm$ 5401 (See Appendix VI).

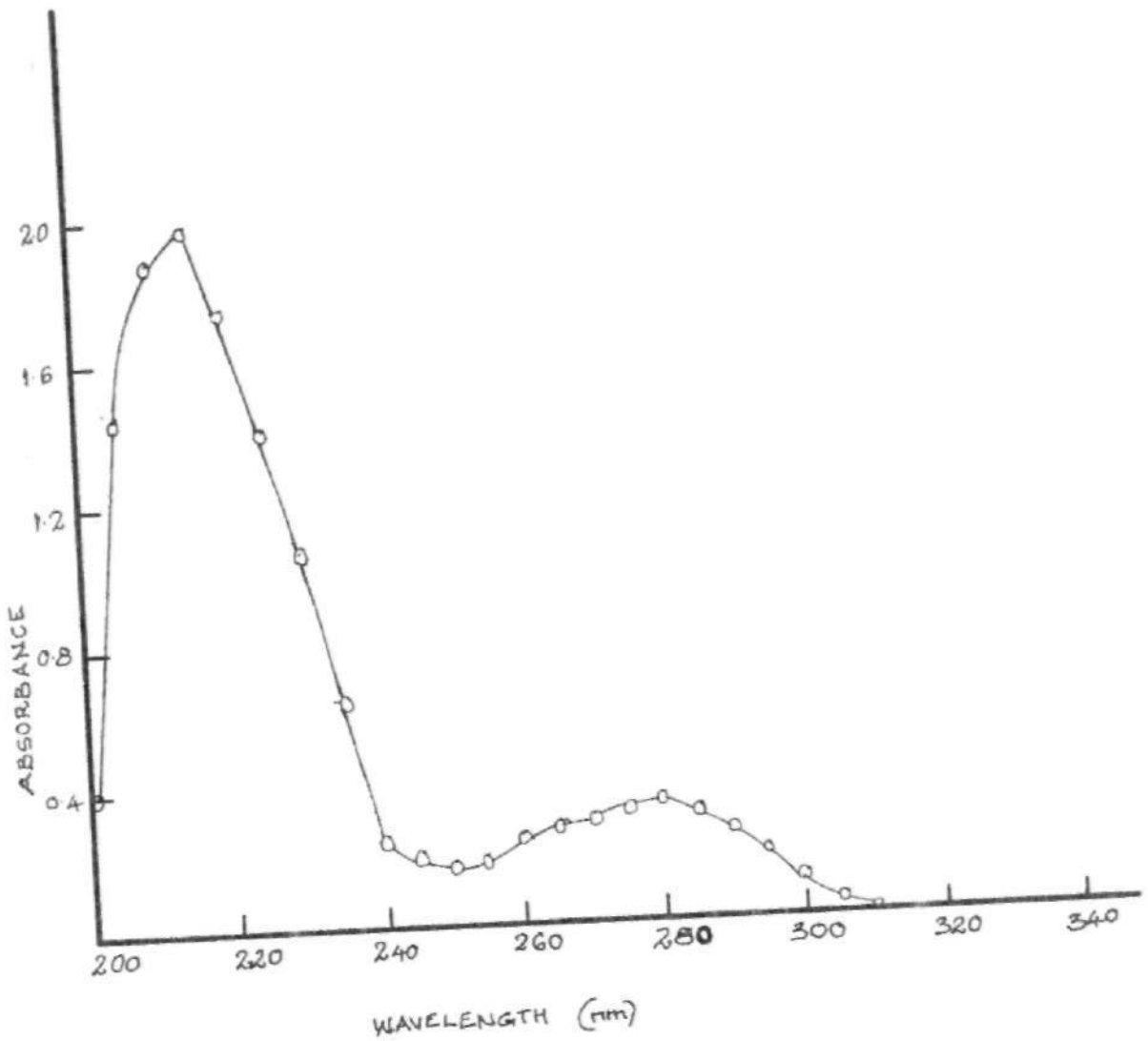


FIG. 13.- Ultra violet spectrum of purified o-diphenolase.

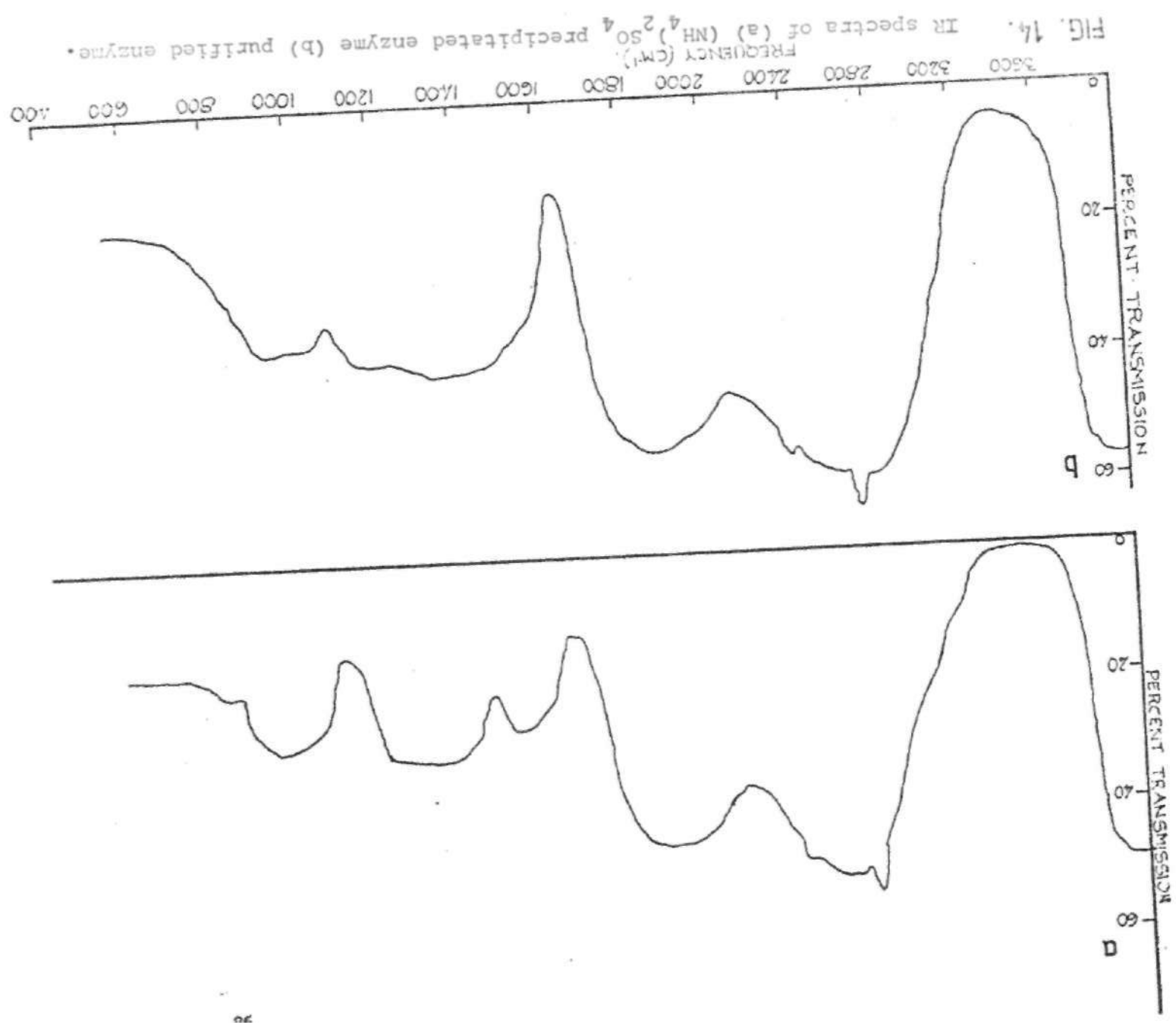


FIG. 14. IR spectra of (a) $(NH_4)_2SO_4$ precipitated enzyme (b) purified enzyme.

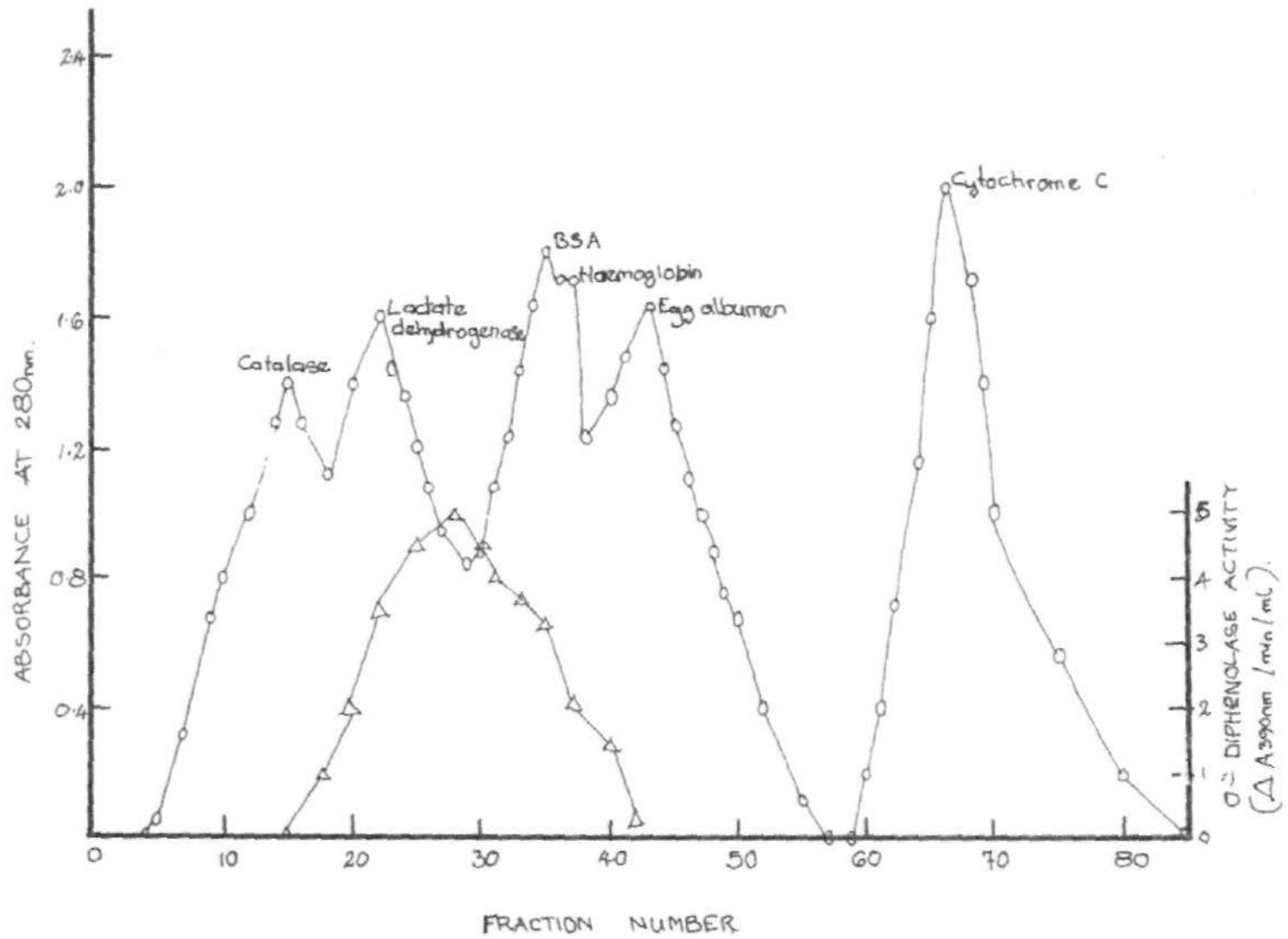


FIG. 15.- Elution profile on sephadex G-200 of purified o-diphenolase (Δ–Δ–Δ) and standard proteins (O–O–O).

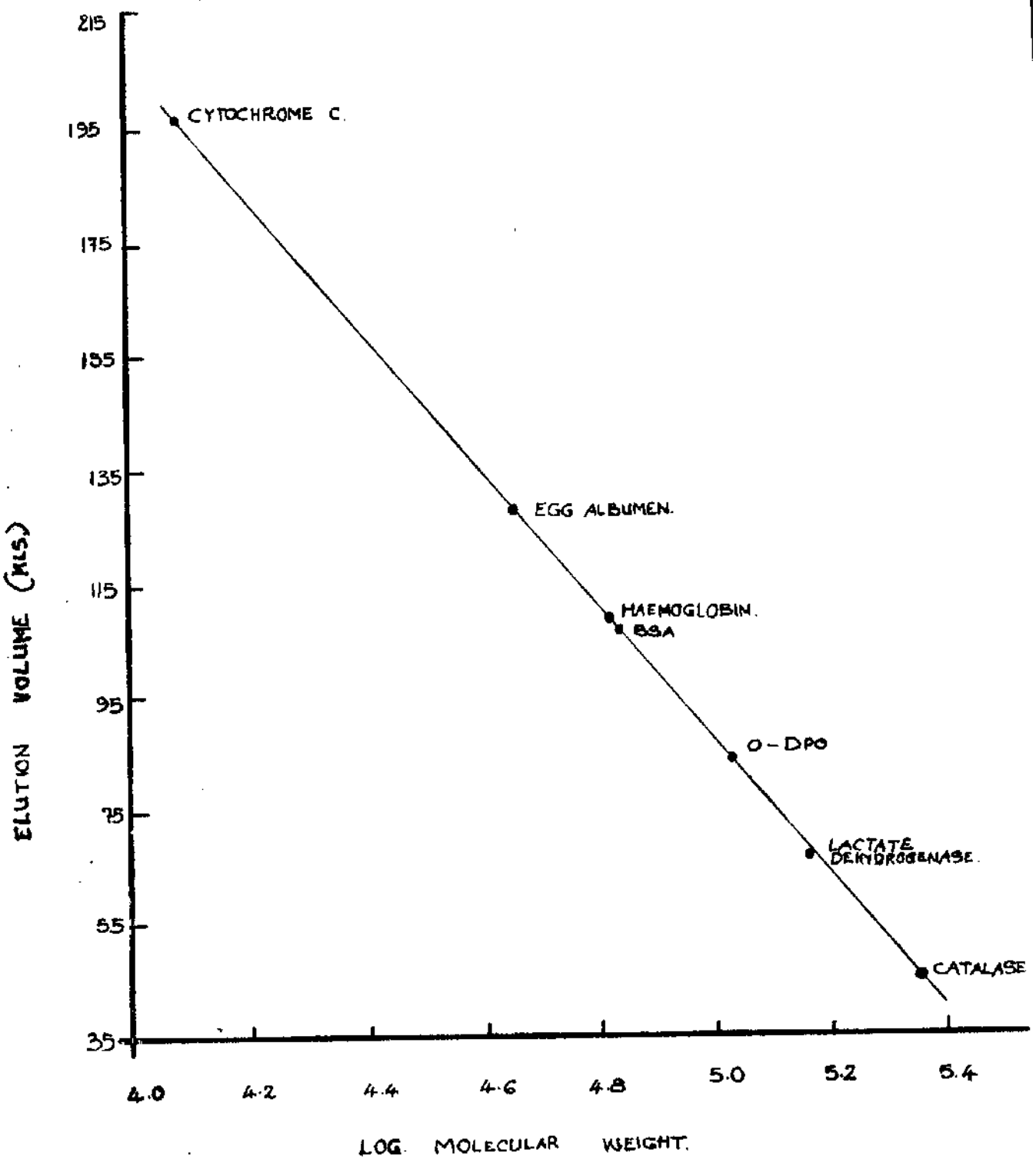


FIG. 16. - Plot of elution volume (mls) versus logarithm of molecular weights of calibrating proteins - for the estimation of molecular weight of o-DPO by gel filtration on a sephadex G-200 column (2.6 x 40cm)

Copper content

The copper content of purified o-diphenolase preparation determined by atomic absorption spectrophotometry was found to be 0.00292mg/mg protein (average of 3 determinations - see Appendix III). Since molecular weight of purified o-diphenolase was estimated to be 107, 333 ± 5401 - which is only 75.6% protein - a copper content of 0.00292 mg/mg protein corresponds to 4 copper atoms (the closest agreement) per molecule of o-diphenolase. Copper content of purified o-diphenolase therefore is 0.22% (see Appendix III).

Amino acid composition

The amino acid composition of the purified *D.rotundata* o-diphenol oxidase shown in Table 8 indicates that there is total absence of proline, ½ cystine and methionine. Tryptophan is known to be lost under the conditions of hydrolysis employed and no attempt was made to determine it by some other method.

Table 8 - Amino acid composition of purified o-diphenolase from D.rotundata cv. Zaria

Amino acid	g/100g protein
Tryptophan	-
Lysine	1.2
Histidine	0.3
Arginine	8.9
Aspartic acid	4.1
Threonine	3.2
Serine	0.9
Glutamic acid	2.7
Proline	0
Glycine	1.2
Alanine	1.2
Cystine(½)	0
Valine	5.3
Methionine	0
IsoLeucine	0.7
Leucine	2.5
Tyrosine	0.5
Phenylalanine	0.9

DISCUSSION

It has been shown that o-diphenolase is greatly involved in the browning reaction of exposed yam surfaces (Adamson and Abigor, 1980). In this study, o-diphenolases were extracted from ten cultivars of four cultivated yam species grown for food in Nigeria, and purified from one of the cultivars (cv. "Zaria"). Yam like any other plant contains a lot of phenolic compounds (Martin and Ruberte, 1976; Tono, 1976). According to Loomis and Battaile, (1966) the presence of phenolic compounds - which combine with the proteins on maceration of the tissues reversibly by hydrogen bonding, and irreversibly by oxidation followed by covalent condensations - makes it impossible to isolate active enzymes by conventional techniques from plant tissues. This problem is made worse in the case of o-diphenolase since the phenolic compounds are its substrates. In this investigation, acetone powder preparation was chosen as the first step of purification in order to remove as much endogenous phenolic substrates from the enzyme as possible. Acetone powder preparation also effects solubilization of membrane-bound o-diphenolase which could also have complicated its isolation (Mayer and Harel, 1979).

Ammonium sulphate fractionation of the crude enzyme extract from Dioscorea rotundata cv. "Zaria" did not increase its purification fold. This observation is in contrast to the observation of Interesse et al, (1980) who reported that treatment of o-diphenolase of wheat with 55% $(NH_4)_2SO_4$ saturation gave a

nine-fold purification and a 3-fold increase in total units of activity, due to release, of latent form of enzyme. Latency of o-diphenolase due to the combination of the enzyme with a diffusable inhibitor have been reported by various authors (Kenten, 1958; Robb et al, 1966 ; Benjamin and Montgomery, 1973; Sato and Hasegawa, 1976; Kahn, 1977). It should be noted however, that what might be considered as "induced" latency is frequently observed after preparing acetone powders (Ben-Shalom et al, 1977). Such latency might well be a result of the acetone treatment rather than a native property of the enzyme.

The purification procedure employed - as described in the "Experimental section" - yielded an enzyme 122 - fold purer than the crude enzyme extract from acetone powder. On disc gel electrophoresis with 7.5% polyacrylamide gel at pH 9.0, a single protein band which moved slightly towards the anode was observed from the purified o-diphenolase preparation. On incubation with catechol, the o-diphenolase could be detected only in the protein corresponding to the stained band. The fact that the disc without any sample showed no band implies that the observed bands are not electrophoretic artifacts. Thus, o-diphenolase from D. rotundata "cv. "Zaria" is made up of a single enzyme. It was also found that electrophoretic patterns of the o-diphenolase from D. rotundata are similar to Solanum melongena o-diphenolase pattern

obtained by Sharma and Ali (1980) and different as compared to electrophoretic patterns of royal Ann cherry (Benjamin and Montgomery, 1973), d'Anjou pears (Halim and Montgomery, 1978) and mango (Park et al, 1980) o-diphenolases. These authors including several others have reported the presence of multiple forms or isozymes of o-diphenolases from different sources including tobacco (Clayton, 1959) and mushrooms (Bouchilloux et al, 1963).

Despite all these ample pieces of evidence, the existence of o-diphenolases in multiple forms still remains enigmatic up till today. According to Mayer and Harel, (1979) it is not certain whether this multiplicity of o-diphenolases is a native 'genuine' phenomenon or artifacts resulting from partial denaturation and fragmentation during purification. For example, the differences in physical and enzymic properties and ratios of cresolase to catecholase activity obtained by Mallette and Dawson, (1949) from mushroom o-diphenolase were ascribed by the investigators to chemical and physicochemical changes in an original single enzyme during the purification process. Bendall and Gregory, (1962) also believed that the various o-diphenolase fractions they obtained from tea leaves were modified forms of one native enzyme. Patil and Zucker, (1965) also concluded that one of the two potato o-diphenolase fractions they obtained contained subunits of the other.

Kertesz and Zito, (1965) obtained from mushroom a single form of active o-diphenolase enzyme in a highly purified form. The reproducibility of the method, and the fact that the amino acid composition of some of the multiple forms obtained earlier by Bouchilloux et al, (1963) was similar, tended to lend support to the assumption that these multiple forms may be artifacts of a single active form.

Changes in the eluting conditions did not alter the elution position of o-diphenolase from DEAE-sephadex column. This shows that the enzyme's conformation may be such that only few prototropic groups are exposed. This possibility seems supported by the fact that the o-diphenolase band obtained in electrophoresis moved only slightly towards the anode even at pH 9.0. Park et al, (1930) also obtained two bands which did not move much towards the anode at pH 8.6 after electrophoresis of mango o-diphenolase.

The amount of o-diphenolase activity (in terms of activity on catechol or catecholase activity) in the ten cultivars of yam were determined from the activities of the crude extracts from their acetone powder preparations. All the D. rotundata cultivars except cv. Orhohozar were found to contain more catecholase activity than cv. Zaria. D.bulbifera cv. Adu was found to have the highest amount of catecholase activity, while the D. alata cultivars had very low amounts.

The values obtained may not represent actual o-diphenolase content in cultivars since only catechol was used as substrate. The low values obtained for some of the cultivars may be higher if a substance like 4-Methyl catechol were used as substrate since according to Duckworth and Coleman, (1970) the rate of oxidation of o-diphenol by o-diphenolase increases with increasing electron withdrawing power of substituents in the para-position. Mayer and Harel, (1979) reported that the level of o-diphenolase in plants often changes markedly during the development of the plant and may be considerably affected by growth conditions. It should be noted that in this investigation, growth conditions and development rates were not taken into consideration in selection of the cultivars used. Marked differences in both the level of o-diphenolase activity and in the content of its substrates have been observed between different cultivars of fruits and vegetables (Harel et al, 1966; Kahn, 1977; Volk et al, 1977). Such differences could be important in commercially exploited plants in which browning is a problem. So in yam processing, such data could be useful for preliminary selections.

o-Diphenolases from all the cultivars were found to have relatively low affinity for catechol (with Km in the order of 10^{-3}M). The affinity of o-diphenolase from cv. Zaria for catechol increased slightly on purification from Km of 13.33mM to Km of 12.50mM.

This is due to the fact that purification increases the sensitivity of an enzyme (Whitaker, 1972). The Km values obtained for the o-diphenolases compare well with values reported for o-diphenolase from cocoa husk (Chaplin, 1978) and o-diphenolase from three cultivars of D.alata (Adamson and Abigor, 1980). o-Diphenolases from plant sources generally have low affinity for the phenolic substrates with high Km values (Mayer and Harel, 1979). However, several authors reported higher affinities for the phenolic substrates in o-diphenolases from some sources - 0.01 - 0.1mM in potato tubers (Alberghina, 1964; Patil and Zucker, 1965), banana (Palmer, 1963), with an exceptionally high affinity (Km ca 1uM) in Papaver somniferum (Asghar and Sidiqi, 1970). Km values vary depending on substrate being used and on the enzyme source. According to Chaplin, (1978) an alkyl group in the 4-position of the o-diphenol (substrate) increases the binding (decreases Km) of the substrate but has minimal effect on the Vmax.

The crude o-diphenolases from cultivars Zaria, Obiaturugo , Oku, Ikpen were found to have broad pH optima (pH 5.5 - 7.2). On purification, cv. Zaria o-diphenolase was found to have pH optimum of 6.8. The other cultivars' o-diphenolases showed two pH optima - one around neutrality, the other around pH 4.5-6.0. All the cultivars' o-diphenolases however showed optimum around neutrality.

These observations agree with reported results of the effect of pH on the activity of o-diphenolase in many fruits. The first report was in 1950 when Asimov and Dawson reported that the enzyme operates best over a pH range of 5.5-7.0, and that there was no marked optimum. Paul and Janave, (1973) also observed maximum activity between pH 6.5 - 7.0 in mango fruit o-diphenolase, using p-cresol and dopamine as substrates, while Park et al, (1980) observed maximum activity between pH 5.5 - 6.0 with catechol as substrate. Two pH optima have also been observed in o-diphenolases from many sources - pH 5.2 and 7.3 for apples (Shannon and Pratt, 1967), pH 5.0 and 7.3 for grapes (Harel and Mayer, 1971) and pH 5.3 and 6.9 for wheat (Interesse et al, 1980). o-Diphenolase systems in fruits such as apricots and prunes (Joslyn and Ponting, 1951), apple (Betrosian et al, 1960), cranberry (Chan and Yang, 1971), clingstone peaches (Wong et al, 1971) and d'Anjou pears (Halim and Montgomery, 1975) have been reported to be most active at neutral pH.

Plots of log Vmax, -log Km and log $\frac{Vmax}{Km}$ versus pH and analyses of these after the method of Dixon and Webb, (1964) revealed that there are two groups on the enzyme with pKs of 4.7 and 7.2 which may be involved in effecting transformation of substrate to product, binding of substrate to the enzyme and preservation of the native conformation of the active site.

Data presented in Table 5 reveal that all the o-diphenolases from the ten cultivars were active towards the o-diphenols and polyphenols but not with monophenols. The results indicate that o-diphenolases of all the ten cultivars of yam studied can not catalyse the oxidation of monophenols to o-diphenols i.e. they lack cresolase activity. This observation compares favourably with observations on the substrate specificities of o-diphenolases from several other plant sources - apple chloroplast (Harel et al, 1965), Clingstone peach (Wong et al, 1971), royal Ann cherry (Benjamin and Montgomery, 1973), green olives (Ben-Shalom et al, 1977a), wheat (Interesse et al, 1980) and mango (Park et al, 1980). All the o-diphenolases from these sources were found to be devoid of cresolase activity. According to Mayer and Harel, (1979) it is difficult to assess whether these represent genuine "native" forms of the enzyme. Mason, (1956) had reported that monophenolase or cresolase activity of o-diphenolase is less stable than the o-diphenolase activity and may be lost as a result of changes in the structure of the protein during its purification. Harel et al, (1956) reported loss of cresolase activity on solubilization of membrane-bound o-diphenolase. Since acetone powder preparation effects solubilization of membrane-bound o-diphenolase (Mayer and Harel, 1979), if any monophenolase activity were present in any of the cultivar o-diphenolase, it may have been lost during acetone powder preparation.

On the other hand, the apparent inability of all the o-diphenolases to oxidise monophenols is in contrast to the broader specificity of the mushroom and potato enzymes (Bouchilloux et al, 1963; Patil and Zucker, 1965).

Data presented in Table 6 show the effect of a number of inhibitors on the crude and purified o-diphenolase from cv. Zaria, with catechol as substrate. In its role during oxidative browning, o-diphenolase presents culinary problems, as the ugly brown polymerized product is associated with off-flavours including bitterness. Prevention of the enzyme activity during processing with safe reagents is thus important in food technology. Data from inhibition studies may also yield information on the active site of the enzyme. Sodium metabisulphite, diethyldithiocarbamate (DEDTC), dithiothreitol (DTT), and cysteine were found to be the most effective, followed by ascorbic acid, 2-mercaptoethanol and potassium cyanide. DEDTC has been shown to complex the copper prosthetic group and to inhibit the o-diphenolase of broad bean (Swain et al, 1966), peaches (Wong et al, 1971), royal Ann cherry (Benjamin and Montgomery, 1973) and mango (Park et al, 1980) even at very low concentrations. Sodium metabisulphite was found to be the most effective inhibitor of mango o-diphenolase, while ascorbic acid was the least potent (Park et al, 1980).

Ascorbic acid acts more as an antioxidant than a true enzyme inhibitor. It reduces the initial product (a quinone) formed by the enzyme before the quinone can undergo secondary reactions to produce brown products characteristic of the browning reaction, and has no effect on the enzyme per se. (Clayton, 1959; Whitaker, 1972). The inhibition shown by the thiol compounds might be due to addition to the quinones to form thioethers and/ or by being reducing agents (Loomis and Battaile, 1966; Mathew and Parpia, 1971) or by combining with the copper required for enzymatic activity (Sharma and Ali, 1980).

Ethylene-diaminetetra acetic acid (EDTA) showed no inhibition under the conditions of assay used. It could therefore be postulated that the copper is deep within the three-dimensional configuration of the protein or is tightly bound to the protein of the enzyme or the stability of the complex of copper ion with the protein is stronger than that of the copper ion and EDTA at pH 6.8. Sharma and Ali, (1980) also found that EDTA did not inhibit o-diphenolase of Solanum melongena at pH 6.5. The inhibition by dithiothreitol (DTT) may be due to the formation of strong chelates with the copper of the enzyme. DTT has been reported to be quite selective for those enzymes which contain copper (II). (Whitaker, 1972).

Since o-diphenolases usually have a low content of cysteine (Jellinck and Smith, 1971) - and the present work seems to support this - the inhibition by p-hydroxy mercuric benzoate and p-chloromercuric benzoate which are SH-reagents, may be due to the heavy metal ion (Hg) present in these reagents. The inhibitory effect of heavy metals depends on replacement of copper from the enzyme (Mathew and Parpia, 1971).

The inhibition by cysteine and ascorbic acid is interesting from a nutritional and processing standpoint since most other inhibitors of o-diphenolase are toxic to man. In addition, cysteine is an important amino acid in the body and ascorbic acid is a vitamin. If a means were available for the transport of these chemicals across the waxy culticular layer of the whole yam tuber or if they are applied to the foliage of the growing yam plant, they could be used to prevent friction discoloration of yams. Such procedures could be used to prevent enzymic browning caused by physical, mechanical and physiological injury in most yams. Several investigators have found L-cysteine to be a potent inhibitor in d'Anjou pears (Halim and Montgomery, 1978), cocoa husk (Chaplin, 1978), Solanum melongena (Sharma and Ali, 1980) and have suggested its use as a means of preventing enzymic browning in these plants. Splittstoesser et al, (1973) and Onwueme, (1976) have reported yams to be lacking in sulfur-containing amino acids. Finding a way of applying this amino acid to yams would not only serve the purpose of preventing enzymic browning but also to supply the sulfur-containing amino acids, which are essential for human nutrition.

L-cysteine, potassium cyanide (KCN) and 2-mercaptoethanol were found to competitively inhibit the purified enzyme. The competitive inhibition by cysteine is similar to the findings of Chaplin, (1978) and Adamson and Abigor, (1980) for cocoa husk and *D. alata* o-diphenolases respectively. Chaplin, (1978) however found cyanide a non-competitive inhibitor, and suggested that there are at least two sites on the enzyme, both probably containing copper; a phenolic substrate binding site and an oxygen substrate binding site. In this investigation, KCN was found to be competitive to the phenolic substrate, catechol. KCN is known to inhibit enzymes containing essential copper (Whitaker, 1972). According to Mayer and Harel, (1979) reagents which react with the copper in o-diphenolase show competitive inhibition with oxygen, inhibition being non competitive towards the phenolic substrate. Since KCN was found to be competitive to catechol in this investigation, it may indicate that o-diphenolase of cv. Zaria has just one binding site for both oxygen and the phenolic substrate which contains copper. The binding of O_2 to this site enables the binding of the phenolic substrate. This is mere speculation and needs further investigation. There is however, a lot of discrepancies in the literature on the number of binding sites of o-diphenolase (Mayer and Harel, 1979).

Nonetheless, the presence of competition between copper chelating agents and the substrate implies that the metal is primarily engaged in the binding of substrate to the enzyme.

Sodium azide was found to be an uncompetitive inhibitor of the purified o-diphenolase. This may be due to the fact that not all the enzyme-substrate (ES) complex formed, proceeded to yield product, some were present as E-S-azide complex which did not break down to yield product. Azide forms complexes with copper (Witaker, 1972). The binding of azide to the ES complex might be through the copper atom of the complex.

When kept at room temperature for two weeks, the purified o-diphenolase lost about 80% of its initial activity. It was also found to be stable when heated at temperatures up to 60^{o}C for 5 minutes. On heating at 70^{o}C for 5 minutes, 54% of its initial activity was lost. The enzyme was however completely inactivated on heating at 100^{o}C for 5 minutes. Heat of inactivation (ΔH) for the purified enzyme, calculated from Arrhenius equation was found to be 26.69 Kcal. between 30--100^{o}C. This high value may be due to the large positive entropy change resulting from the opening up and unfolding of the enzyme molecule during denaturation.

The o-diphenolase assay procedure employed is not only sensitive and rapid, but measures the initial oxidation of the substrate without including complex polymerization reactions or allowing for the inhibition of the enzyme by the products of the reaction.

During the enzymatic oxidation of catechol by the purified enzyme, rate of oxidation was linear for only the first 2 minutes, after which the activity fell. This phenomenon has been termed reaction inactivation and has been investigated and discussed by various authors (Asimov and Dawson, 1950; Krueger, 1962). According to Pierpoint et al, (1977) and Chaplin, (1978), this inactivation may be as a result of irreversible products inhibition due to the o-quinone structure produced combining with free lysine or arginine groups on the enzyme. Linearity in mushroom has been observed to be for 2 to 3 minutes (Smith and Krueger, 1962), 2 minutes in royal Ann cherry o-diphenolase (Benjamin and Montgomery, 1973), while that of potato was for 5 minutes (Friedman and Daron, 1977). Whitaker, (1972) also reported a decrease in absorbance at 395nm which he attributed to further reactions (probably non enzymatic) which eventually resulted in the formation of an insoluble brown pigment.

The purified o-diphenolase from cv. Zaria was found to be 24.2% carbohydrate. The enzyme is thus a glycoprotein. o-Diphenolases from other sources have also been found to contain non-amino acid components. Yunusov et al, (1975) found that 26% of o-diphenolase from cotton consist of an arabinose, xylose, galactose and glucose-containing polysaccharides. They concluded that the carbohydrate moiety is attached through the hydroxyl of

a C-terminal serine of the cotton enzyme. Balasingam and Ferdinand, (1970) also observed that ribonucleic acid accounts for half of the weight of purified potato tuber o-diphenolase.

The estimated molecular weight (MW) of purified o-diphenolase of cv. Zaria was 107, 333 ± 5401. This value compares well with molecular weights of the predominant forms of mushroom o-diphenolase which have been reported to be between 116,000 and 120,000 (Smith and Krueger, 1962; Bouchilloux et al, 1963; Kertesz and Zito, 1965). Lewis and Dawson, (1948) also reported a MW value of 100,000 for the highly purified enzyme from Lactarius piperatus. Reported values for molecular weight of o-diphenolase from various sources cover a wide range. Harel and Mayer, (1966) reported MWs of 30000 - 40000, 60000 - 70000 and 120000 - 130000, for the three forms of o-diphenolase they isolated from apple fruits, and these were shown to undergo interconversions. Multiplicity of MWs was also observed by Demenyuk, (1974) in apple o-diphenolase; this led him to conclude that the high MW forms are transformed into lower MW forms during ripening and storage of the fruit. Gregory and Bendall, (1966) also concluded that the native tea o-diphenolase has a MW of 144000 ± 16,000, the multiplicity observed being the result of partial degradation and tanning reactions during isolation. From the foregoing, it is clear that the o-diphenolase from cv. Zaria was not fractionated into low molecular weight forms during isolation.

Strothkamp et al, (1976) suggested that the mushroom enzyme contains two types of polypeptide chains or subunits, a heavy (H with MW ca 43000) chain, and a light (L with MW ca 13400) chain differing in their amino acid composition. If this is so, then the purified o-diphenolase from cv. Zaria may be a tetramer of quaternary structure L_2H_2. This, however, needs to be verified experimentally.

The copper content of purified o-diphenolase was found to be 0.22%. Since the MW was determined to be 107,333 ± 5401, this value corresponds to four copper atoms per molecule of the o-diphenolase. This value is close to the value of 0.21% obtained by Bouchilloux et al, (1963) for mushroom o-diphenolase based on MW of 119,000. The distribution of the copper atoms within the molecule is not yet certain. Numerous reports suggest a copper content of one atom per polypeptide chain or subunit (Robb et al, 1965; Kertesz and Zito, 1965; Vaughan et al, 1975). According to Makino et al, (1974) the functional unit of the enzyme contains a pair of copper ions. Whether such a pair is attached to a single polypeptide chain or to two idential or different subunits is not yet clear (Mayer and Harel, 1979). More work therefore needs to be done to ascertain the subunit structure of D.rotundata o-diphenolase.

The ultraviolet (UV) spectrum of the purified o-diphenolase exhibited a typical protein absorption spectrum (high absorption around 215nm due to the vibrations of the peptide bonds of the protein moiety) with an additional shoulder at 280nm due to aromatic amino acids. The spectrum is similar to the spectra reported for mushroom o-diphenolase (Kertesz and Zito, 1965) and for Solanum melongena o-diphenolase (Sharma and Ali, 1980). However, in the present preparation, the shoulder was observed at 280nm instead of 290nm and 285nm reported for the mushroom and solanum melongena enzymes respectively. Royal Ann cherry o-diphenolase obtained by Benjamin and Montgomery, (1973) also showed a typical maximum at 280nm.

Suggestions for further research

It would be interesting to carry out studies on the distribution of the enzyme in the yam tuber, the amino acid sequence and the 3-dimensional structure of the purified o-diphenolase from cv.Zaria.

Also, the structure of the active site of o-diphenolase and the ligands present in it need to be elucidated.

Purification and characterization of the o-diphenolases from the other cultivars would also be necessary.

In view of the observations on inhibition of o-diphenolase by short chain peptides and by specific proteins (Walker, 1970; Mayer and Harel, 1979) and since oligopeptides such as glutathione are known

to form complexes with copper, and therefore to inhibit o-diphenolase, (Clayton, 1959, Mathew and Parpia, 1971; Sharma and Ali, 1980) it may be suggested that short chain peptides should be surveyed with the aim of finding non-toxic inhibitors of the enzyme for possible use in yam processing industry.

APPENDIX I

Purification of o-diphenolase

Definitions

One unit of enzyme activity is equivalent to that amount of enzyme that can cause an increase in absorbance at 390nm of 0.001 per minute under the described assay conditions.

Activity = Change in absorbance at 390nm/min/ml

Total activity = Total volume x Activity/ml

$$\text{Specific activity} = \frac{\text{Activity (units/ml)}}{\text{protein (mg/ml)}}$$

$$\text{Recovery or yield \%} = \frac{\text{Total activity of purer preparation}}{\text{Total activity of crude preparation}}$$

$$\text{Fold purification} = \frac{\text{Specific activity of purer preparation}}{\text{Specific activity of crude preparation}}$$

Determination of ammonium sulphate fractionation range

The crude enzyme extract after extraction of acetone powder with 0.2M potassium phosphate buffer pH 7.5 was divided equally into 7 test-tubes. Solid $(NH_4)_2SO_4$ was added to give 10%, 20%, 35%, 50%, 65%, 80%, and 95% saturation at room temperature. Successive precipitates were collected by centrifugation 30 minutes after each addition of salt, redissolved in 0.1M potassium phosphate buffer pH 6.8, and assayed for o-diphenolase activity and protein content as previously described. The specific activities towards catechol were calculated and plotted against % $(NH_4)_2SO_4$ saturation (Fig.1.). From the shape of the curve, 20-85% $(NH_4)_2SO_4$ fractionation range was selected.

Table 9 - Determination of o-diphenolase content (units) in cultivars

Cultivar (o-diphenolase source)	Wt. of fresh tuber used (gms)	Wt. of acetone powder formed (gms)	Volume of crude extract of o-DPO from Acetone powder 10gm (ml)	o-Diphenolase (catecholase) activity towards catechol (30mM) per ml of crude extract (units) x 10^{-3}. (A390nm/min/ml)	o-Diphenolase activity per 10gm acetone powder (units) x 10^{-3}	β-Diphenolase (catecholase) activity per gm acetone powder (units) x 10^{-3}	Tuber wt. giving 1 gm acetone powder (gm)	o-Diphenolase (catecholase) activity per gm fresh tuber wt. (units) x 10^{-3}.
Dioscorea rotundata								
1 Zaria (Zaria)	439.450	110.400	21.5	41.0	881.50	88.150	3.981	22.1450
2 amara "	437.900	100.000	21.0	56.0	1176.00	117.600	4.379	26.8550
3 orhohozar (Benin)	569.300	184.750	10.0	28.0	280.00	28.000	3.081	9.0867
4 omi "	590.400	174.400	21.0	51.0	1071.00	107.100	3.385	31.6360
5 obiaoturugo (Umuahia)	519.300	136.750	21.0	68.0	1428.00	142.800	3.797	37.6040
Dioscorea alata								
6 Igiehua (Benin)	570.250	112.500	23.0	10.9	250.70	25.070	5.069	4.9459
7 Mbala (Umuahia)	651.650	149.050	21.0	10.0	210.00	21.000	4.372	4.8032
Dioscorea cayenesis								
8 Oku (Umuahia)	315.450	102.550	21.5	27.5	591.25	59.125	3.076	19.2210
9 Ikpen (Benin)	389.500	97.550	20.0	16.2	324.00	32.400	3.993	8.1146
Dioscorea bulbifera								
10 Adu (Umuahia)	234.00	61.600	15.0	104.0	1560.00	156.000	3.799	41.0660

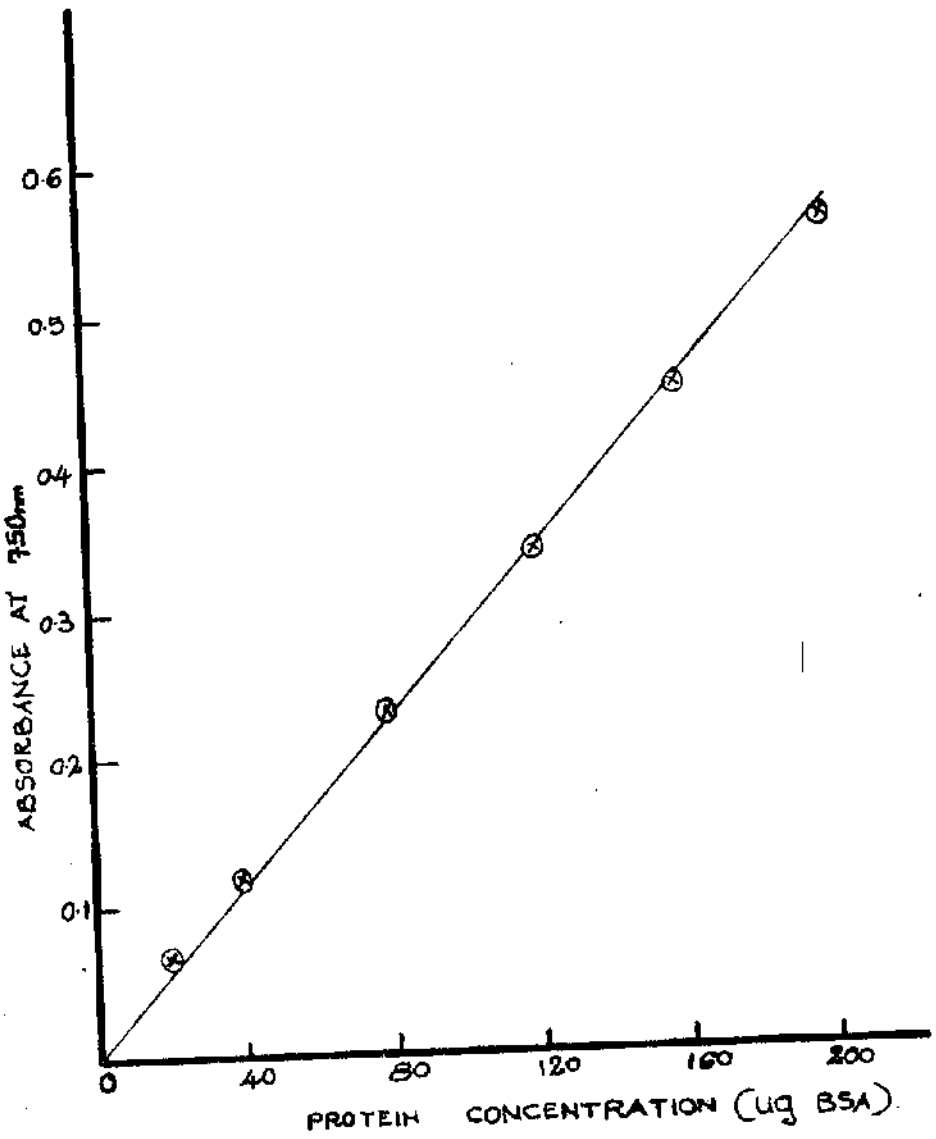


FIG. 18:- Standard curve for the determination of protein content.

APPENDIX III

The Folin-Lowry method of protein assay
(Lowry, et al, 1951)

Reagents:

A) 50% Folin ciocalteau reagent (1:1% H_2O)

B) 2% w/v sodium potassium tartarate

C) 1% w/v $CuSO_4$

D) 2% $NaCO_3$ in 0.1N NaOH

Bovine serum albumin (BSA) stock solution - 200ug/ml

Procedure:

1 Standard solutions of BSA of concentration range 0-200ug/ml were prepared in different test-tubes

2 To 50ml of D was added 1ml of B + C (0.5ml of each). Five mls of the resulting 'alkaline solution' was added to each tube, mixed thoroughly and let to stand for 10 minutes at room temperature.

3 Then 0.5ml of the diluted Folin-Ciocalteau reagent (immediately prepared) was added to each tube, shook vigorously, and allowed to stand for 30 minutes for colour development.

4 Absorbance of each tube contents was read at 750nm against a blank which had no BSA and plotted against protein (BSA) concentration (Fig. 18). Extracts solutions were tested in the same way and the corresponding protein concentrations were read from the calibration curve.

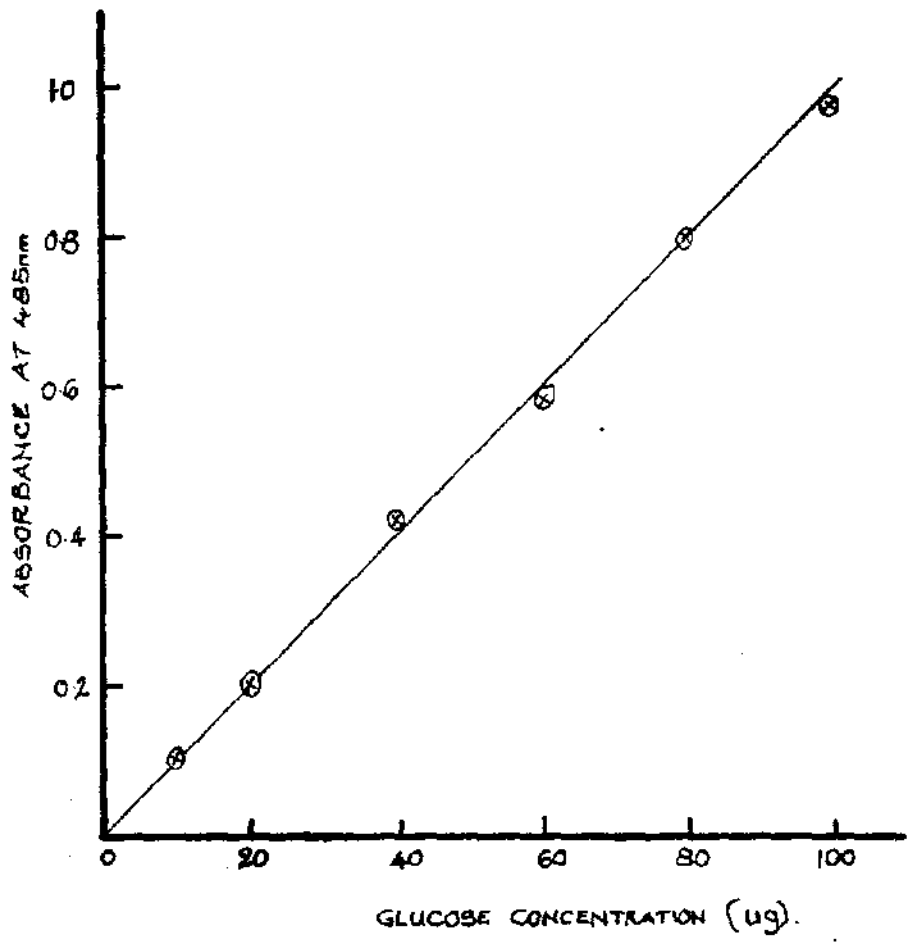


FIG. 19. - Standard curve for estimation of total carbohydrate in enzyme preparations.

Appendix III conts.

Estimation of total carbohydrate by the phenol-sulphuric acid Method

The method of Dubois et al, (1956) was followed with some slight modifications.

Reagents:

5% aqueous phenol (v/v) solution.

Concentrated H_2SO_4 grade 98%

Standard glucose solution (0.1mg/ml or 100µg/ml)

Procedure:

Aliquots containing 10-100 ug of the standard glucose solution were pipetted into clean reaction-tubes and the volume made up to 1.0ml with distilled water. One millilitre of the 5% phenol solution was added to each tube followed by rapid addition of 3.0ml of the concentrated H_2SO_4. The tubes were then covered with stoppers and boiled in a water bath for 20 minutes. The tubes were cooled at room temperature and the absorbance of the solutions was read at 485nm against a blank which contained no glucose.

A graph of absorbance against concentration of glucose gave the calibration curve shown in Figure 19. The solution of the enzyme preparations were treated in the same way and the corresponding concentrations read from the calibration curve.

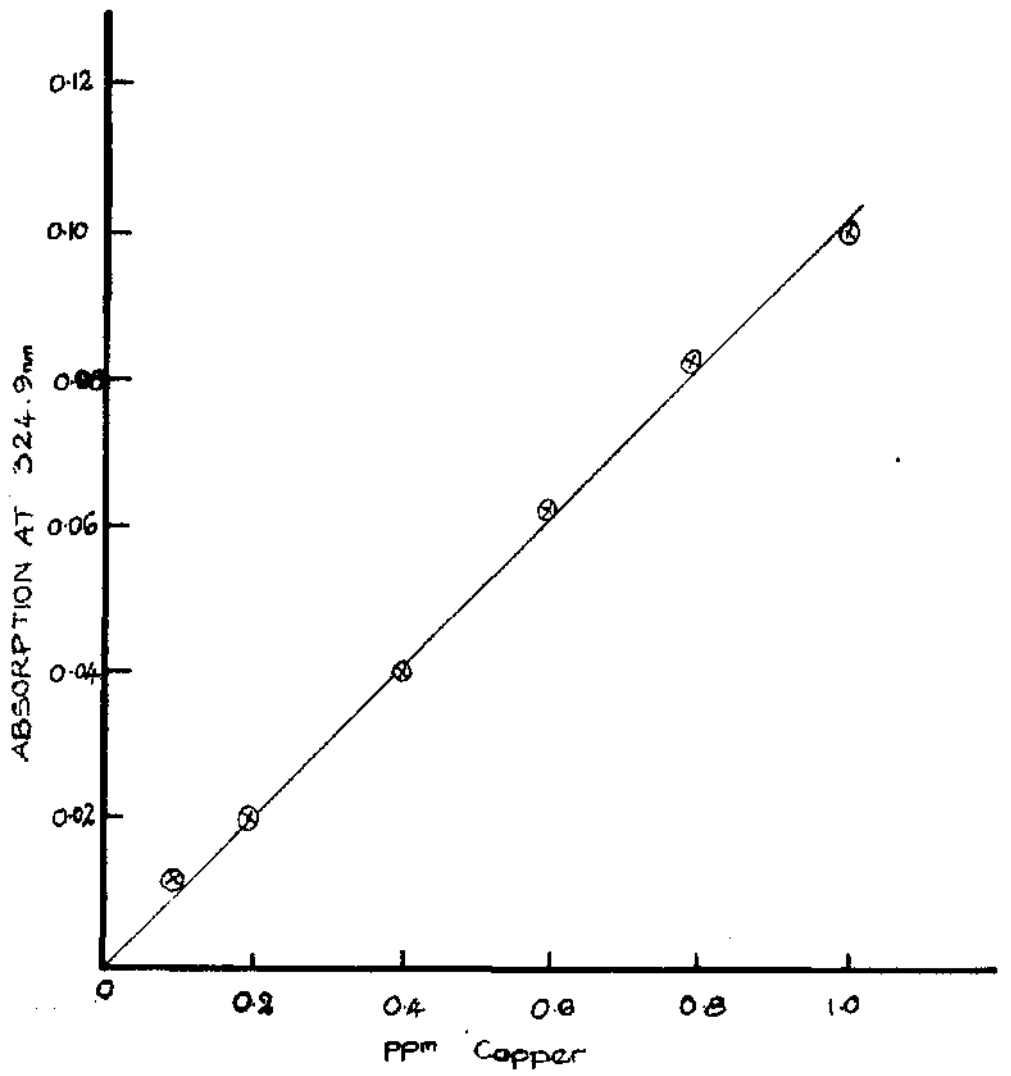


FIG. 20. - Standard curve for estimation of copper content.

Appendix III conts.

Estimation of % protein and % total carbohydrate of pure o-diphenolase preparation

Protein content (Lowry et al, 1951) of pure o-DPO preparation

= 1625ug/ml

Total carbohydrate content = 520ug/ml

Dry weight of 4mls of pure o-DPO preparation = 8600µg = 8.6mg

Protein content = 1625µg/ml = 6500µg/4mls = 6.5mg

$\therefore$ % Protein = $\frac{6.5}{8.6}$ x 100 = 75.6%

Total carbohydrate content = 520µg/ml

= 2080µg/4mls

= 2.08mg

$\therefore$ % Total carbohydrate = $\frac{2.08}{8.60}$ x 100 = 24.2%

Estimation of copper content of purified o-diphenolase

Copper content of 3 different preparations (P_1,P_2,P_3) of pure o-DPO were estimated from the standard plot of absorbance at324.9nm versus ppm copper (Fig. 20).

Sample	Atomic Absorption at 324.9nm	Protein content(µg/ml)
P_1	0.230	77.40
P_2	0.310	107.60
P_3	0.160	55.0

Appendix III conts.

Sample P_1

From standard curve (Fig 20) copper content = 0.23ppm

This is equivalent to 0.23g copper per 1 million ml of extract

$= 0.23 \times 10^{-3}$mg copper/ml

Protein content = 77.40×10^{-3}mg/ml

∴ Copper content = 0.23×10^{-3}mg/77.40×10^{-3}mg protein

= .00297mg/mg protein.

Sample P_2

Copper content = 0.31ppm $\equiv 0.31 \times 10^{-3}$mg/ml

Protein content = 107.60×10^{-3} mg/ml

∴ Copper content = 0.31mg/107.60mg protein = .00288mg/mg protein.

Sample P_3

Copper content = 0.16ppm = 0.16×10^{-3}mg/ml

Protein content = 55×10^{-3}mg/ml

∴ Copper content = 0.16mg/55mg protein = .00291mg/mg protein

Average copper content = $\frac{.00297 + .00288 + .00291}{3}$

= .00292mg/mg protein.

∴ Copper content = 0.00292mg/mg protein of o-DPO.

Appendix III conts

But o-DPO is 75.6% protein i.e. for every 100mg of o-DPO, 75.6mg of it is protein.

∴ 0.00292 x 75.6mg copper corresponds to amount of copper in 100mg of o-DPO = 0.22mg.

∴ Copper content of pure o-DPO is 0.22%

Determination of number of copper atoms per molecule of o-DPO

Mean molecular weight of o-DPO = 107333.33 (see appendix VI)

Copper content = 0.22%

∴ Copper content per molecule = $\frac{0.22}{100}$ x 107333.33gm

= 236.13gm

Atomic weight of copper = 63.6

∴ Number of copper atoms per molecule of o-DPO = $\frac{236.13}{63.6}$

≃ 4 (the nearest whole number)

APPENDIX IV

Determination of kinetic parameters

Table 10 - Determination of Km at different pHs

	pH4			pH5		pH6		pH6.8		pH 8.0	
S (mM)	$\frac{1}{S}$ (mM^{-1})	V (ΔA_{390nm} /min /ml)	$\frac{1}{V}$	V	$\frac{1}{V}$	V	$\frac{1}{V}$	V	$\frac{1}{V}$	V	$\frac{1}{V}$
10	.100	.625	1.6	6.02	0.166	6.99	0.143	8.93	0.112	0.50	2.00
15	.067	.90	1.11	7.14	0.140	8.47	0.118	11.49	0.087	0.70	1.43
20	.050	1.18	0.85	8.33	0.120	9.62	0.104	12.50	0.080	0.91	1.10
25	.040	1.35	0.74	9.10	0.110	10.42	0.096	13.50	0.074	1.05	0.95
30	.033	1.59	0.63	10.00	0.100	11.36	0.088	14.70	0.068	1.20	0.83

Table 11 - Determination of Km and Vmax in presence and absence of Inhibitors at pH 6.8

Inhibitor	$-\frac{I}{Km}$ (mM^{-1})	$-\frac{I}{Km_I}$ (mM^{-1})	$\frac{I}{Vmax}$	$\frac{I}{Vmax_I}$	Type of inhibition
Control (No I)	0.08	-	0.05	-	-
KCN (1mM)	-	0.014	-	0.05	Competitive
" (0.667mM)	-	0.019	-	0.05	"
Cysteine (0.1mM)	-	0.040	-	0.05	Competitive
" (0.167mM)	-	0.030	-	0.05	"
2-mercapto-ethanol (0.167mM)	-	0.060	-	0.05	Competitive
NaN_3 (2mM)	-	0.170	-	0.106	Uncompetitive

Calculation of Ki

NaN_3 - Inhibition is uncompetitive

Ki can be calculated from

$Vmax = Vmax_I \left(1 + \frac{[I]}{Ki} \right)$ (Whitaker, 1972).

where Vmax = maximum velocity in absence of inhibitor

$Vmax_I$ = maximum velocity in presence of inhibitor

$[I]$ = Inhibitor concentration.

or from

$$Km = Km_I \left(1 + \frac{[I]}{Ki}\right)$$

Substitution for Km and Km_I at $[I]$ = 2mM

$$12.5 = 5.88 \left(1 + \frac{2}{Ki}\right)$$

$$Ki = \underline{1.776mM}$$

KCN, Cysteine and 2-mercaptoethanol inhibited o-DPO competitively.

For linear competitive inhibition.

$$Km_I = K_m\left(1 + \frac{[I]}{Ki}\right)$$

For KCN, at $[I]$ = 1mM

$$71.43 = 12.5 \left(1 + \frac{1}{Ki}\right)$$

$$71.43\ Ki - 12.5\ Ki = 12.5$$

$$58.93\ Ki = 12.5$$

$$Ki = \underline{0.210mM}$$

OR At $[I] = 0.667mM$

$$52.63 = 12.5 \left(1 + \frac{0.667}{Ki}\right)$$

$$\underline{Ki = 0.210mM}$$

For Cysteine,

at $[I] = 0.1mM$

$$25.0 = 12.5 \left(1 + \frac{0.1}{Ki}\right)$$

$$Ki = \underline{0.100mM}$$

at $[I] = 0.167mM$

$$33.33 = 12.5 \left(1 + \frac{.167}{Ki}\right)$$

$$Ki = \underline{0.100mM}$$

For 2-mercapto ethanol,

at $[I] = 0.167mM$

$$16.36 = 12.5 \left(1 + \frac{.167}{Ki}\right)$$

$$Ki = \underline{0.502mM}$$

Table 12 - Effect of varying substrate concentration on o-DPO activity

$[S]$ (mM)	V (ΔA_{390} nm/min/ml)
5.0	4.0 ± 0.4
10.0	8.0 ± 0.8
15.0	11.8 ± 0.6
20.0	15.6 ± 0.8
25.0	19.2 ± 0.8
30.0	20.1 ± 0.8
35.0	20.0 ± 0.8
40.0	20.0 ± 1.2

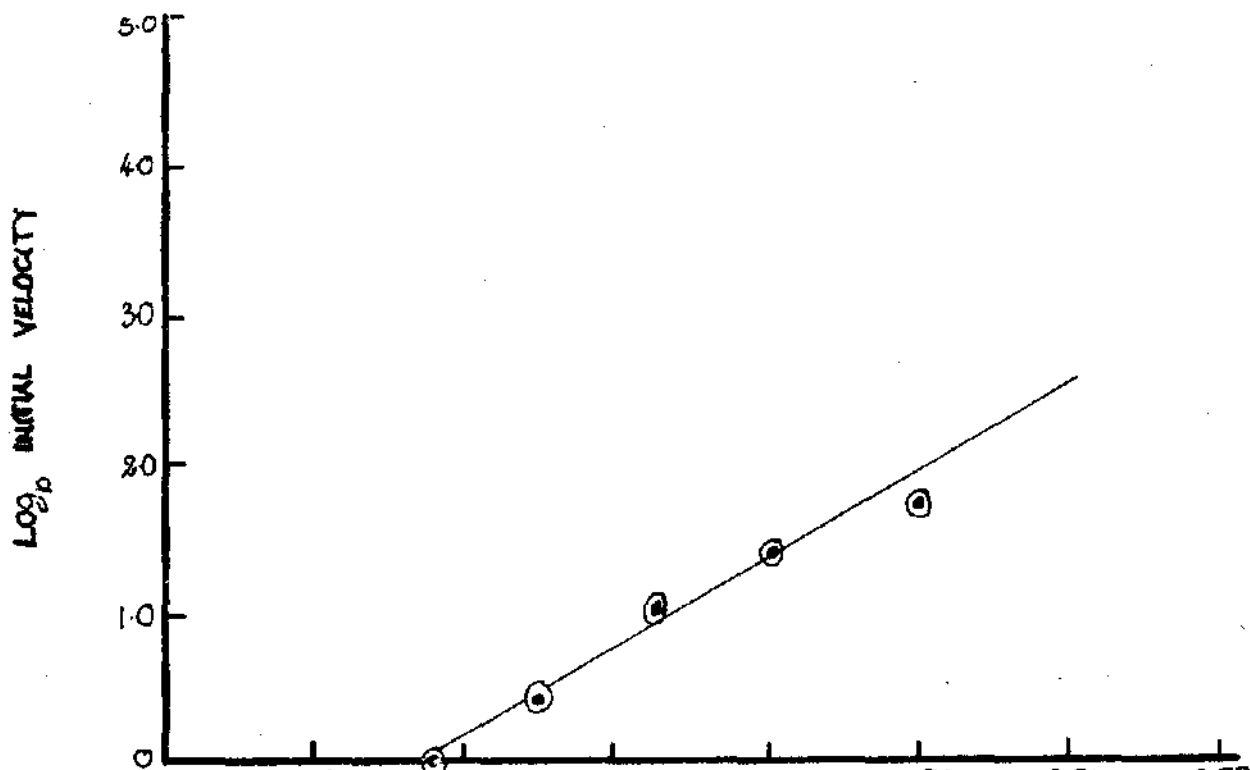


FIG. 17.- Arrhenius plot for the reaction of o-DPO with catechol (30mM) in 0.1M phosphate buffer pH 6.8 reaction mixture.

Slope of curve = $E/2.303R$

E = Energy of activation
R = Gas constant (1.986cal/deg./mole)

APPENDIX V

Table 13 - Effect of temperature on the stability of the enzyme protein

Temperature (^{o}C)	^{o}A	$\frac{I}{T}(^{o}A) \times 10^{2}$	Initial velocity (ΔA_{390}nm/min/ml)	V per 10mls	$Log_{10}V$
26	299	0.334	5.00	50.0	1.6990
30	303	0.330	5.00	50.0	1.6990
40	313	0.319	4.90	49.0	1.6902
50	323	0.310	5.00	50.0	1.6990
60	333	0.300	5.20	52.0	1.7160
70	343	0.291	2.30	23.0	1.3617
80	353	0.283	1.30	13.0	1.1139
90	363	0.275	0.25	2.5	0.3979
100	373	0.268	0.10	1.0	0.0000

Calculation of Heat of Inactivation (ΔH) of o-DPO

Slope of Arrhenius plot (Fig. 17) = $\frac{E}{2.303\ R}$ (Plummer, 1971)

Slope = $\frac{2.2 - 0.5}{.00305 - .002765} = \frac{1.7}{.000285} = 5964.9122 = \frac{E}{2.303\ R}$

where R = Gas constant = 1.986 cal/deg/mole

E = Energy of activation

$\therefore$ E = 5964.9122 x 2.303 x 1.986 cals/mole = 27282.063 cals/mole

E = ΔH + RT where ΔH = Heat of inactivation

T = Temperature in ^{o}A. = $299^{o}A$

$\Delta H = E - RT$ = 27282.063 - (1.986 x 299) cals.

= 27282.063 - 593.814 cals = 26688.249 cals

Heat of inactivation of o-DPO (ΔH) = 26688.249 cals/mol.

APPENDIX VI

Determination of molecular weight by gel filtration on a sephadex G-200 column (2.6 x 40cm)

The following values were obtained from 3 different runs

1) 112,800

2) 107,200

3) 102,000

Mean $(\bar{x})$ = 107,333.333

Molecular wt. (x)	Deviation from the mean $(x-\bar{x})$	$(x - \bar{x})^2$
112,800	+ 5466.67	29884480.000
107,200	- 133.33	17776.888
102,000	+ 5333.33	28444408.000
		58346664.888

$$\text{Standard deviation} = \sqrt{\frac{(x - \bar{x})^2}{n - 1}}$$

$$= \sqrt{\frac{58346664,888}{2}}$$

$$= 5401.23$$

$\therefore$ Molecular weight = 107,333.33 + 5401.23

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