STUDIES ON OXIDASE ACTIVITY IN POTATO TUBERS

IV. A QUANTITATIVE METHOD FOR THE DETERMINATION OF PEROXIDASE*

by

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INTRODUCTION

In a previous communication[1], we described a method for determining the tyrosinase activity of white potatoes by macerating potato dice in the presence of aqueous o-phenylenediamine. The tyrosinase formed a strongly fluorescent orange-yellow compound that could be extracted with organic solvents, and whose concentration could be determined spectrophotometrically or fluorometrically.

In the current studies we report that the same general method can be applied to the determination of potato peroxidase activity. When white potatoes are macerated in a WARING blendor, and a suitable aliquot of the resultant homogenate added to aqueous o-phenylenediamine plus hydrogen peroxide, a fluorescent orange-yellow compound is formed. Advantage is taken of the fact that with the addition of hydrogen peroxide, readily determinable color and fluorescence results with the use of such dilutions of the potato homogenate that only insignificant tyrosinase activity occurs.

EXPERIMENTAL

Materials

Potatoes are washed, peeled, and cut into dice of approximately 1 ml volume, which are then washed briefly under running water.

For the preparation of the homogenate, a suitable amount of dice (40 g except where otherwise noted) is added to enough water in a WARING blendor to give a volume of 300 ml, and blended for 60 seconds at high speed. At the end of this time, the speed of the blendor is reduced. While the mixture is stirring slowly, a few drops of caprylic alcohol are added to destroy the foam, and a 20 ml aliquot of the homogenate is pipetted from the blendor. Under these conditions a representative sample is obtained. The homogenate is prepared immediately prior to use.

Where indicated, blanched dice were prepared by placing the dice, in a colander, into boiling water for stated periods of time, immediately after which the dice were cooled in ice water.

Purified horse-radish peroxidase was prepared by the method of WILLSTAETTER[2].

25 g of o-phenylenediamine dihydrochloride (EASTMAN KODAK) are dissolved in water to a final volume of 2 liters of solution whose p_H has been adjusted to 5.0 with sodium hydroxide.

The following materials were also used:

30% H_2O_2 — superoxol, Merck, A.R.;

Acetone — Merck, U.S.P.;

Normal butanol — Merck, Reagent.

Methods

100 ml of the o-phenylenediamine solution are added to 200 ml of water, stirring slowly in a beaker. To the resultant solution are added 0.7 ml of superoxol, then 20 ml of the homogenate. All

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materials, when used, were at room temperature (22–26° C). Stirring is continued for $4^1/_2$ minutes, at the end of which time 50 ml of the reaction mixture is removed, and after 5 minutes of reaction time, are added to 50 ml of acetone to stop the reaction. 5 ml of the acetone mixture are pipetted into 95 ml of water and 100 ml of butanol in a separatory funnel. After vigorous shaking, the butanol layer is separated, filtered through a WHATMAN $\frac{11}{11}$5 filter paper and read in a KLETT-SUMMERSON colorimeter, using standard KLETT test tubes, with the number 42 blue filter against a butanol blank.

For the fluorescence determination, 2 ml of the butanol filtrate are pipetted into 48 ml of butanol and mixed thoroughly. The fluorescence of the solution is determined on a PFALTZ and BAUER fluorophotometer against a standard of 1 part of fluorescein to 4 million parts of 0.01 N NaOH.

For comparative purposes, the purpurogallol peroxidase assay was sometimes carried out on aliquots of the same batch of homogenate used for the o-phenylenediamine test. The technique followed was a modification of WILLSTAETTER's method[2] along the lines suggested by STERN and KEGELES[3]. In general this method depends upon the extraction by ethyl acetate of purpurogallin formed from pyrogallol, and the photometric determination of the optical density of the extract with correction for the tyrosinase blank.

Results

To determine whether or not this o-phenylenediamine reaction was peroxidase-catalysed, pure horse-radish peroxidase was reacted with o-phenylenediamine and hydrogen peroxide. Significant color and fluorescence were obtained only in the presence of all three components.

Varying quantities of a solution of the purified horse-radish peroxidase were assayed using both the pyrogallol and the o-phenylenediamine methods. Results are shown in Table I.

TABLE I

COMPARATIVE REPRODUCIBILITY OF PYROGALLOL AND O-PHENYLENEDIAMINE METHODS

ml Enzyme	O-Phenylene-diamine color*	% Deviation from mean	O-Phenylene-diamine Fluorescence*	% Deviation from mean	Pyrogallol color*	% Deviation from mean
0.2	75	0.8	30	3.3	190	2.9
0.5	76	0.5	28	3.3	180	2.5
1.0	82	8.4	27	6.6	170	7.9
2.0	75	0.8	31	6.6	197	7.4
3.0	70	7.6	29	c	186	0.9
Mean	75.6	3.6	29	3.9	184.6	4.3

* Expressed as optical density calculated for one ml of enzyme solution.

It will be seen that there is a close correspondence between the two methods. Through standardization with purpurogallin of the optical density of the extracts of the pyrogallol reaction mixture, it was determined that the optical density of the butanol extracts of the o-phenylenediamine reaction mixture on the KLETT-SUMMERSON colorimeter could be converted into WILLSTAETTER P.Z. by the following formula:

$$\text{P.Z.} = \frac{\text{D}}{1.54 \times \text{mg of sample}}$$

Where D is the optical density of the butyl alcohol extract of the o-phenylenediamine reaction mixture.

o-Phenylenediamine Peroxidase Assay as Applied to the Potato Tuber

To determine whether the proportionality between the amount of peroxidase and the color development found with the purified horse-radish enzyme would be found in

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the potato tuber, 5, 10, 20, 40, 60 and 80 grams of Green Mountain potato dice were chosen at random from a large batch of dice and homogenized in a WARING blendor with sufficient water to make a total volume of 300 ml. 20 ml aliquots of the homogenates were assayed for peroxidase activity by the o-phenylenediamine method. The results are shown in Figure I.

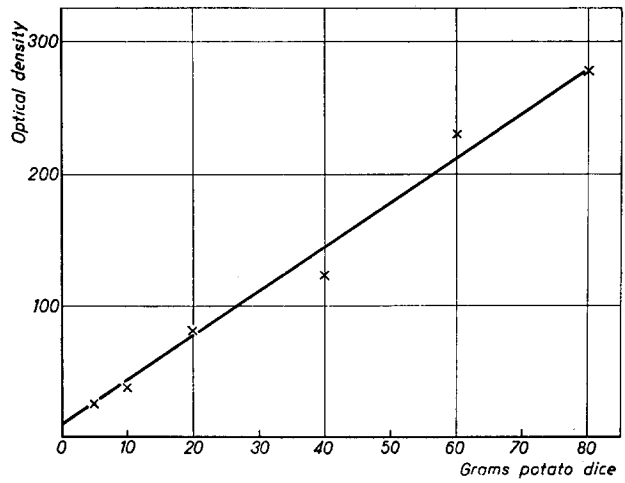

Fig. 1

It will be seen that the color formation is in direct proportion to the amount of peroxidase present.

Reaction Time

The influence of the reaction time on the development of color and fluorescence was studied. The results are shown in Figure II. (For comparative purposes a reaction time curve with the purified peroxidase is shown.)

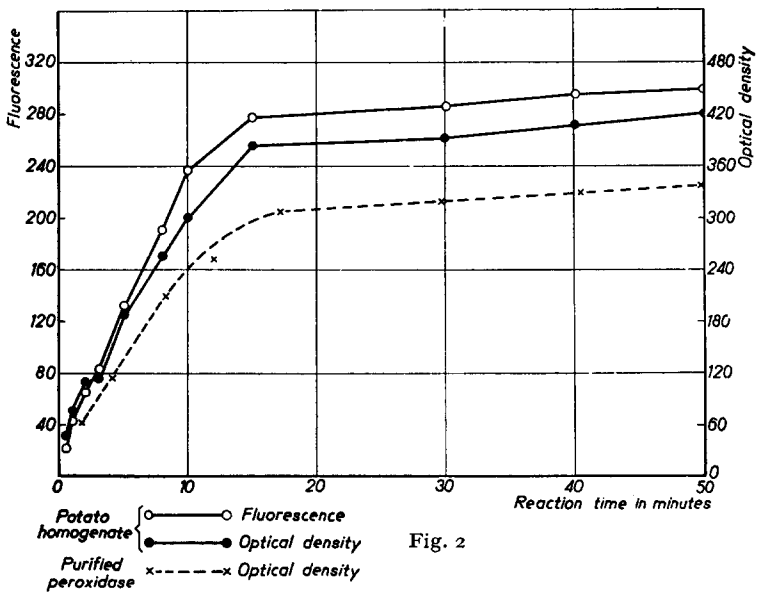

Fig. 2

It will be seen that the formation of color and fluorescence is, in its early stages, linearly proportional to time, falling off rather sharply after 15 minutes.

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Reaction Temperature

The influence of temperature on the peroxidase o-phenylenediamine reaction was studied in respect to both color and fluorescence in 14 determinations at temperatures ranging from 10 to 90° C. The temperatures indicated are those of the reaction mixture. Homogenization was carried out at ordinary temperature (22–26° C). Reagent blanks were run at each temperature, using completely blanched potatoes instead of enzymatically active potatoes. Results are shown in Figure III.

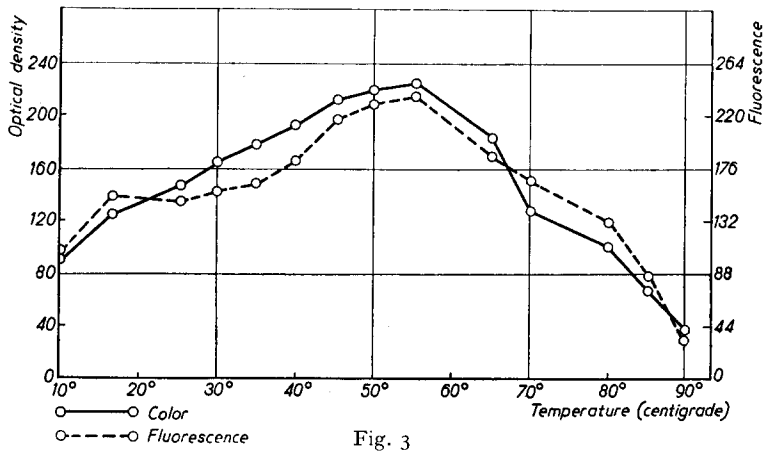

Fig. 3

It will be seen that maximum color and fluorescence occurs in the vicinity of 55° C, but that the peroxidase reaction shows marked persistence even at quite high temperatures

Blanching

The influence on the color formation of blanching for periods up to 90 seconds was studied with both the o-phenylenediamine method and the WILLSTAETTER pyrogallol method. The results are shown in Figure IV.

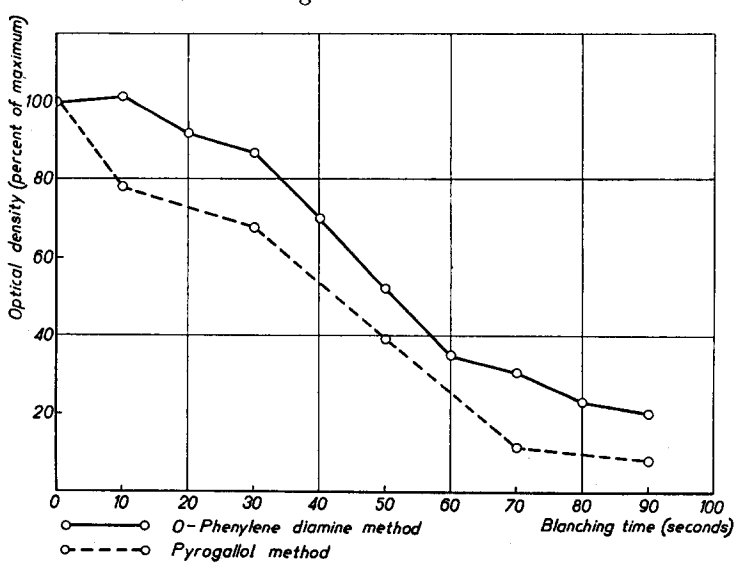

Fig. 4

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It will be seen that a close parallel exists between the two methods, and that the peroxidase is substantially inactivated in 90 seconds blanching.

Correlation of Peroxidase and Tyrosinase

Using the o-phenylenediamine method, the peroxidase content of potatoes was compared with the tyrosinase content as determined by the o-phenylenediamine method previously described[1]. In these experiments, various batches of Idaho and Green Mountain potatoes were assayed for their tyrosinase and peroxidase content. Separate assays were made of the peel, pith and cortex portions of the potato. The results are shown in Figure V.

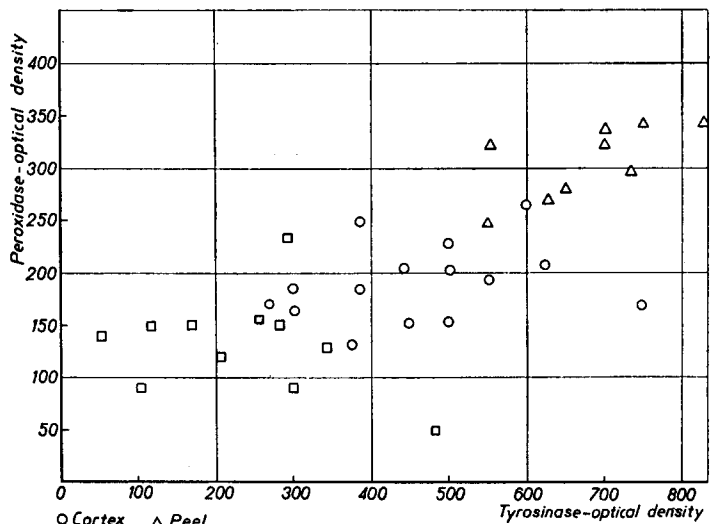

Fig. 5

It will be seen that the peroxidase and tyrosinase show a substantial correlation — highest in the peel and lowest in the pith of the respective potato.

The calculation of the coefficient of correlation[4] between the tyrosinase and peroxidase as determined by the o-phenylenediamine method indicate a positive correlation of 0.76.

Comparison of the mean enzyme activities of peel, pith and cortex are shown in Table II.

TABLE II

PEROXIDASE AND TYROSINASE IN PITH, PEEL AND CORTEX

	Mean Readings (Optical Density)	
	Peroxidase	Tyrosinase
Peel	307	682
Cortex	189	477
Pith	132	247

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DISCUSSION

Our experiments indicate that the o-phenylenediamine method is in reproducibility and precision, of the same order as the classical method of WILLSTAETTER in its more recent modification. In our opinion the o-phenylenediamine method also has certain advantages over the pyrogallol method.

Since the o-phenylenediamine is a much more stable substance than pyrogallol, it is not necessary to run a "substrate control" with each determination. A single substrate control with the o-phenylenediamine will be sufficient for a large number of determinations since this reagent will remain unchanged even after 9 hours standing at room temperature and several days in the ice-box. Pyrogallol, on the other hand, auto-oxidizes rapidly, particularly under the influence of light.

When the WILLSTAETTER method is applied to potatoes, it is necessary to run a tyrosinase control, using no hydrogen peroxide. This control optical density must be deducted from the optical density obtained with hydrogen peroxide to obtain a measure of the peroxidase activity. In the o-phenylenediamine method the amount of oxidation by tyrosinase under conditions of the experiment is virtually negligible (a fraction of 1 %) and for all practical purposes may be disregarded.

If dilution of the original butanol extract is omitted, the fluorometric method can be used to determine very small peroxidase activities.

We have made some attempts to characterize the colored product formed in the peroxidase-o-phenylenediamine reaction, and to compare it with the compound formed by tyrosinase[1]. We have been able to isolate red birefringent needles from the reaction mixture of purified peroxidase, water and o-phenylenediamine. Similar crystals were isolated from the tyrosinase-o-phenylenediamine reaction mixture. Very dilute solutions of the compounds show strong fluorescence. Their color and fluorescence are reversibly discharged by sodium hydrosulfite, being restored on shaking with air. Above p_H 6 both solutions become yellow in color. Below this point the color is orange-red, becoming increasingly red at low p_H levels.

Several statements have appeared in the literature dealing with the formation of a phenazine through the action of peroxidase and hydrogen peroxide on o-phenylenediamine. The compound isolated here shows properties not inconsistent with the known properties of phenazines, but we have not completely characterized it.

In an earlier report on the determination of tyrosinase action in potato by use of o-phenylenediamine[1], the possibility was suggested that the colored compound formed might be "tyrophenazine", formed by the combination of the "red body" of tyrosine oxidation with o-phenylenediamine.

Continuation of the investigation of this "tyrophenazine" has not as yet uncovered any properties that are significantly different from the peroxidase-o-phenylenediamine compound. Melting point tests proved inconclusive because of gradual but extensive decomposition. Spectrophotometric curves of the two compounds show a general similarity. It is conceivable that the o-quinone formed by the action of tyrosinase on dopa does not condense directly with o-phenylenediamine, but rather oxidizes a molecule of o-phenylenediamine in a manner similar to the peroxidase-hydrogen peroxide oxidation of o-phenylenediamine. The resultant molecule, possibly o-phenylenediimine can then combine with a molecule of o-phenylenediamine to give a phenazine.

As indicated in the experimental section, the method works satisfactorily with

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solutions of purified peroxidase. Whether or not the use of o-phenylenediamine can be applied generally to peroxidase determinations in tissues can be decided, of course, only by experimentation, but the method offers promise of a wide applicability.

RÉSUMÉ

1. Description d'une méthode pour la détermination quantitative de la peroxydase, basée sur la réaction de la peroxydase sur la o-phénylènediamine en présence d'eau oxygénée dans des conditions bien déterminées. La réaction est arrêtée par addition d'acétone, le mélange réactionnel extrait par le butanol; ce dernier est soumis à la colorimétrie et à la fluorométrie.

2. Les résultats obtenus par cette méthode sont en excellent accord avec ceux obtenus par la méthode au pyrogallate de Willstätter. La fidélité des deux méthodes est la même, mais la sensibilité et la commodité sont nettement plus grandes avec la méthode à l'o-phénylènediamine.

3. Dans les conditions réalisées, l'intensité de la coloration et celle de la fluorescence croissent proportionnellement au temps pendant 15 minutes environ puis diminuent rapidement.

4. L'optimum de température pour la réaction est voisine de 60°.

5. La comparaison entre l'activité de la tyrosinase et celle de la peroxydase dans les différentes couches de différentes pommes de terre par les méthodes à l'o-phénylènediamine, montre une corrélation très nette entre ces deux activités.

6. L'étude de l'influence du temps de blanchîment montre une diminution constante dans l'activité de la peroxydase, avec conservation d'un résidu faible d'activité après 90 secondes.

7. La substance formée par l'action de la peroxydase sur l'o-phénylènediamine semble être une phénazine. Sa caractérisation définitive cependant n'a pas encore été faite.

SUMMARY

1. A method is described for the quantitative determination of peroxidase in which o-phenylenediamine is reacted upon by peroxidase in the presence of hydrogen peroxide under standard conditons. The reaction is stopped by the addition of acetone, the mixture extracted with butanol, and the butanol extract assayed colorimetrically and fluorometrically.

2. Results by the o-phenylenediamine method correspond closely with the Willstaetter pyrogallol procedure. Reproducibility is comparable in the two methods, whereas sensitivity and ease of assay is substantially greater witn tne o-phenylenediamine procedure.

3. Under the conditons of assay, the amount of color and fluorescence that is developed is linearly proportional to time for some fifteen minutes, and thereafter shows a sharp decline.

4. The temperature optimum for the reaction was in the vicinity of 60° C.

5. Comparison of the tyrosinase and peroxidase activities of the pith, peel and core of various potatoes by the o-phenylenediamine methods showed a substantial positive correlation between the two activities.

6. Studies of the effect of the blanching time show a steady decline in peroxidase activity leaving a small but persistent residue after 90 seconds.

7. The compound formed by the activity of peroxidase on o-phenylenediamine appears to be a phenazine. Full characterization, however, has not yet been achieved.

ZUSAMMENFASSUNG

1. Eine Methode zur quantitativen Peroxydase-bestimmung wird beschrieben, bei der man Peroxydase in Anwesenheit von Wasserstoffsuperoxyd unter standardisierten Bedingungen auf o-Phenylendiamin einwirken lässt. Die Reaktion wird durch Zufügen von Azeton beendet, das Gemisch mit Butanol extrahiert, und der Butanolextrakt colorimetrisch und fluorometrisch bestimmt.

2. Die Resultate mit der o-Phenylendiaminmethode stimmen innerhalb enger Grenzen mit der WILLSTAETTER'schen Pyrogallolmethode überein. Die Reproduzierbarkeit ist bei beiden Methoden vergleichbar, während die Empfindlichkeit und Leichtigkeit der Bestimmung bei der o-Phenylendiaminmethode wesentlich grösser ist.

3. Unter den Bestimmungsbedingungen ist die Stärke der entwickelten Farbe und Fluoreszenz der Zeit linear proportional während ca. 15 Minuten und fällt danach scharf ab.

4. Das Temperaturoptimum für die Reaktion liegt in der Umgebung von 60° C.

5. Die Vergleichung der Tyrosinase- und Peroxydaseaktivitäten von Mark, Schale und Kern verschiedener Kartoffeln mit den o-Phenylendiaminmethoden zeigte eine stark positive Korrelation zwischen beiden Aktivitäten.

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6. Versuche über die Wirkung der Bleichungsdauer zeigen eine stetige Abnahme der Peroxidaseaktivität, wobei nach 90 Sekunden nur eine kleine, aber beständige Restaktivität bleibt.

7. Die durch die Einwirkung von Peroxydase auf o-Phenylendiamin gebildete Verbindung scheint ein Phenazin zu sein. Volle Charakteristik wurde jedoch noch nicht erreicht.

REFERENCES

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