STUDIES ON OXIDASE ACTIVITY IN POTATO TUBERS

II. o-PHENYLENEDIAMINE AS A FLUOROMETRIC REAGENT *

by

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In a previous communication (I), a method has been described for the determination of phenol oxidase activity in potato tubers by macerating a representative sample in presence of o-phenylenediamine and determining the degree of color formation photoelectrically after adding a portion of the solution to acetone and filtration. The acetone precipitates colloidal impurities and also serves to stabilize the color for at least several hours. It was found that the degree of color formation was an inverse function of the blanching time, and that the extent of color varied with the temperature and p_H of the experimental system.

The color formation was tentatively attributed to the trapping by the o-phenylenediamine of an orthoquinoid intermediate of the tyrosine-tyrosinase reaction; the orange-colored substance was termed „Tyrophenazine" on the assumption that it represents a phenazine-like condensation product (See I). Solutions of „Tyrophenazine" in 80% acetone solution were found to be markedly green fluorescent when exposed to the filtered radiation of a Mercury high-pressure burner (360 mμ). This fluorescence, however, was somewhat irregular and faded rapidly. When an aqueous solution of „Tyrophenazine" was extracted with n-butyl alcohol, an extract was obtained which maintained its fluorescence intensity for at least half an hour.

The addition of one part of ethyl acetate to two parts of butanol in the extraction mixture did not affect the stability of the fluorescence and substantially improved the ease of separation of the extract from the aqueous phase. Larger proportions of ethyl acetate caused progressive fading of the fluorescence.

It was found that under these conditions the degree of fluorescence closely paralleled the color formation. The color formation in comparable volumes was essentially identical whether it was measured in the 80% acetone solution as described in our previous report, or in the butanol-ethyl acetate extract.

EXPERIMENTAL

50 grams of potatoes were macerated in a Waring Blendor for 2 minutes in the presence of 10 ml of a 1% solution o-phenylenediamine hydrochloride, and 240 ml of water. The mixture was strained through cotton into a 500 ml Erlenmeyer flask, and allowed to stand for a total of 15 minutes at room temperature (25—30° C) from the

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beginning of blending. At the end of 15 minutes, 5 ml of the solution were pipetted into a separatory funnel containing 50 ml of an extracting mixture consisting of 2 parts of normal butanol and 1 part of ethyl acetate as well as 40 ml of water. The mixture was vigorously shaken for approximately a half minute and then permitted to separate. The aqueous layer was run off, and the organic solvent layer filtered through Whatman # 5 filter paper. The filtrate was colored clear yellow.

1 part of the filtrate was mixed with 24 parts of the butanolethyl acetate mixture prior to determination of the fluorescence. This dilution corresponds to a concentration of o-phenylenediamine of the order of 1—1.5 gamma per ml.

The intensity of the fluorescence of the diluted solution was measured against that of a solution of fluorescein in N/100 NaOH (1 : 4,000,000) with a Pfaltz and Bauer fluorophotometer. A wave-length of 440 millimicrons was employed for the determination of fluorescence. The unknown fluorescence was read after adjusting the deflection of the galvanometer to 100 scale divisions with the fluorescein standard in the light path.

In the blanching experiments dice were cut to 1 cubic centimeter size. A colander was placed in a large boiling water bath. The dice were dropped into the colander which was withdrawn from the bath after a measured number of seconds. The volume of the bath must be sufficiently large so that the boiling temperature is maintained despite the addition of the cold dice.

Steam blanching was carried out by placing separated dice on a cheese cloth holder in a glass desiccator and passing steam through the cloth from below. The steam circulated freely throughout the glass vessel and escaped through an open hole at the top.

p_H determinations were made with a Beckman p_H meter; parallel colorimetric determinations with a Klett-Summerson photoelectric colorimeter with a blue filter (#42).

RESULTS

To determine the reproducibility of the method, seven 50-gram samples taken from a large uniform batch of potatoes of the Mohawk variety were blended in the presence of o-phenylenediamine and air according to the above procedure. Table I shows the variations in fluorescence intensity in the seven samples of Mohawk potatoes.

TABLE I

REPRODUCIBILITY OF FLUORESCENCE

Sample	Intensity of Fluorescence (Scale Division)	Deviation from Mean	% Deviation
1	64	—4	—5.9
2	66.5	—2.5	—3.7
3	72	4	5.9
4	68.5	0.5	0.7
5	68	0	0
6	66	—2	—2.9
7	70.5	2.5	3.7
Average	68		

It will be seen that the method is reproducible within some 6% of the mean.

The increase of fluorescence in the „Tyrophenazine" reaction with reaction time was compared with the corresponding increase in color formation. The relative color and fluorescence curves are shown in Figure 1.

It will be seen that the curve of fluorescence increase closely parallels the degree of color formation and attains a virtual asymptote beyond 15 minutes.

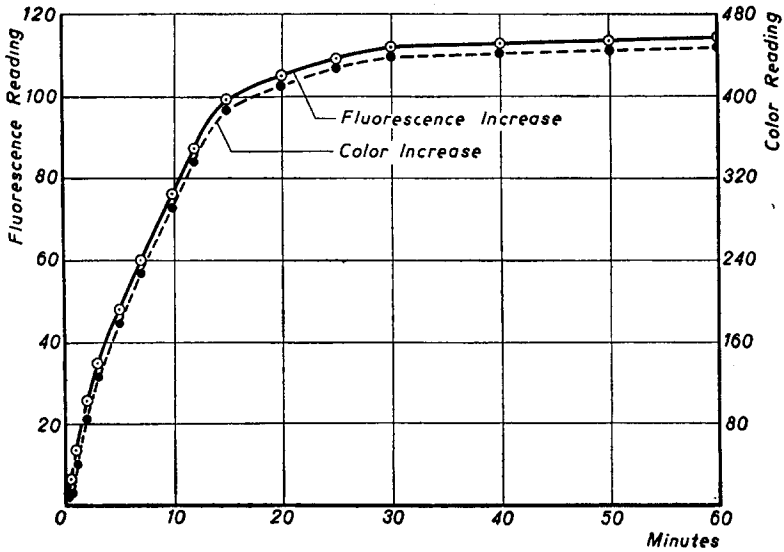

Fig. 1. Comparative Increase of Fluorescence and Color Formation in „Tyrophenazine" Reaction with Time.

The effect of blanching boiling water on fluorescence and color formation was studied with the aid of 4 batches of potatoes representing, in the order of greatest to least original reading, Idaho, Sebago, Irish Cobbler, and Green Mt. potatoes.

The observations made are shown graphically in Figure 2.

It will be seen that color formation and fluorescence are again closely parallel and that the degree of each is an inverse function of the blanching time, both fluorescence and color being abolished after a 60-second blanching period. A 2½ to 3 minute steam blanch was required to achieve complete enzymatic inactivation.

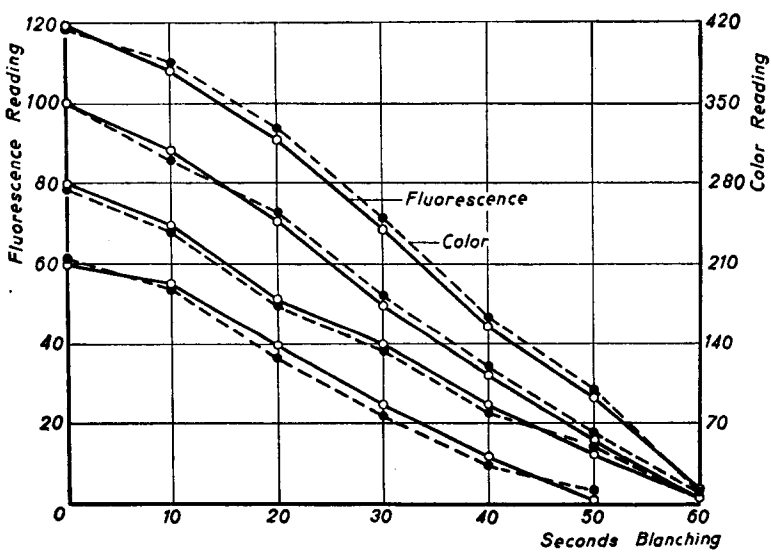

Fig. 2. Effect of Blanching on Fluorescence and Color Formation in „Tyrophenazine" Reaction.

The influence of the hydrogen ion concentration was studied in respect to fluorescence and color formation. Varying quantities of dilute phosphoric acid and sodium hydroxide were added to the mixture of the potato dice and o-phenylenediamine solutions at the beginning of blending, the hydrogen ion concentration being determined on the

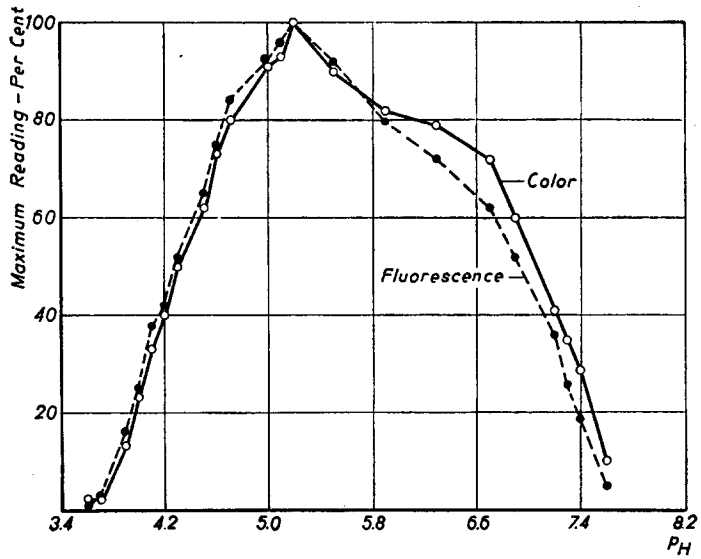

Fig. 3. Influence of p_H on the Fluorescence Intensity.

filtrate. Comparative fluorescence and color intensity are expressed for each p_H in terms of percentages of the maximum which occurred at p_H 5.2 when no acid or alkali was added to the mixture. The results are shown in Figure 3.

It will be seen that both color and fluorescence are progressively diminished by the addition of acid or alkali, and that inactivation becomes complete at p_H 3.7 and p_H 7.9.

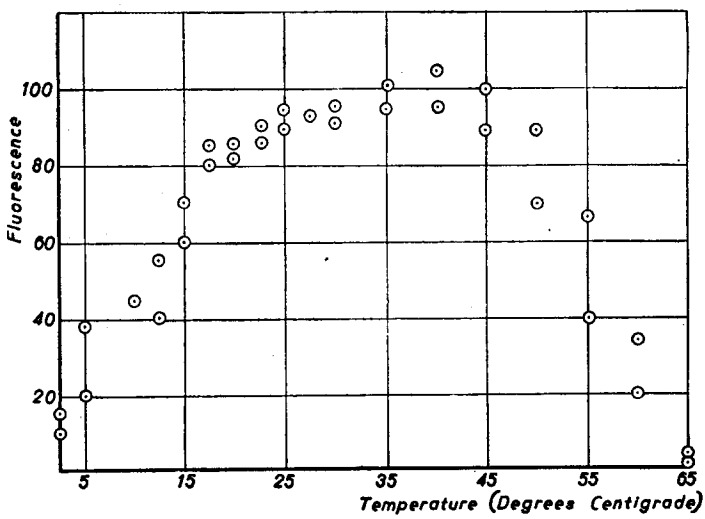

Fig. 4. Influence of Temperature on the Fluorescence Intensity.

The effect of temperature on fluorescence and color formation was studied with two batches of Idaho potatoes with the results shown in Figure 4.

It will be seen that the degree of fluorescence formation is a function of the temperature, reaching its maximum in the vicinity of 40° C. and being progressively diminished as the temperature is increased or decreased beyond this point. The temperature 25 to 30° at which the experiments were conducted differs but slightly from the maximum.

DISCUSSION

The close parallelism observed in these experiments between the fluorescence and the color formation produced by the addition of o-phenylenediamine suggests that a single colored compound or type of compound is involved which becomes fluorescent under appropriate conditions.

The high sensitivity of the fluorometric method when applied to the oxidase activity of potatoes makes it of special value where only traces of such enzymatic activity are likely to be present, as, for example, in blanched or dehydrated materials.

SUMMARY

1. A method is described for determining the phenol oxidase activity of potatoes by measuring the green fluorescence extracted with an organic solvent from potato maceration juice prepared in the presence of a dilute solution of o-phenylenediamine.

2. The degree of fluorescence, measured against a standard of fluorescein, closely parallels the degree of color formation resulting from the o-phenylenediamine-potato juice reactions.

3. The fluorescence, paralleling color, is in inverse relation to the blanching time and is completely extinguished in a 60-second blanch in boiling water. Like color formation, the fluorescence intensity reaches a maximum at approximately 35—43° C. and at a p_H in the range of 4.9 to 5.5.

4. The method was found to be reproducible within about six percent. It is highly sensitive so as to reveal even traces of enzymatic activity.

5. It is assumed that the fluorescent material is identical with the orange-red compound formed under *the same* conditions and which has been provisionally designated as *"Tyrophenazine"*.

RÉSUMÉ

1. Description d'une méthode pour la détermination de l'activité phénoloxydasique des pommes de terre en mesurant la fluorescence verte extraite du jus des pommes de terre macérées, préparé en présence d'une solution diluée de o-phénylène-diamine, au moyen d'un solvant organique.

2. Le degré de fluorescence, mesuré par rapport à la fluorescéine standard, varie à peu près comme le degré de la coloration résultant des réactions du jus de pommes de terre en présence de l'o-phénylène-diamine.

3. La fluorescence, de même que la coloration, est en relation inverse à la durée de blanchiment et elle s'éteint complètement par un blanchiment à l'eau bouillante de 60 secondes. De même que pour la coloration, l'intensité de la fluorescence atteint son maximum entre 35 et 43° environ et à un p_H variant de 4.9 à 5.5.

4. On a trouvé que la méthode peut être reproduite dans les limites de 6% environ. Elle possède une sensibilité telle que même des traces d'activité enzymatique peuvent être révélées.

5. On présume que la matière fluorescente est identique au composé rouge-orange fourni dans les *mêmes* conditions et auquel on a donné provisoirement le nom de *„tyrophénazine"*.

ZUSAMMENFASSUNG

1. Beschreibung einer Methode zur Bestimmung der Phenoloxydase-Aktivität von Kartoffeln bestehend in der Messung der grünen Fluoreszenz des durch ein organisches Lösungsmittel extrahierten Stoffes, der in Kartoffelmazerationssaft bei Gegenwart einer wässerigen o-Phenylendiaminlösung gebildet wird.

2. Der Grad der Fluoreszenz, gegen eine Standard-Fluoresceinlösung gemessen, gleicht dem Grad der Farbstoffbildung in den o-Phenylendiamin-Kartoffelsaft-Reaktionen.

3. Die Fluoreszenz, ähnlich der Färbung, steht in ungekehrtem Verhältnis zur Bleichdauer und wird durch eine Bleichung von 60 Sekunden in kochendem Wasser ausgelöscht. Aehnlich wie bei der Farbstoffbildung, erreicht die Fluoreszenz-Intensität ihr Maximum bei ca. 35—43° und bei einem p_H zwischen 4.9 und 5.5.

4. Es wurde festgestellt, dass die Methode innerhalb einer Grenze von ca. 6% reproduzierbar ist. Ihre Empfindlichkeit ist so gross, dass selbst Spuren von enzymatischer Aktivität festgestellt werden können.

5. Es wird angenommen, dass der fluorescierende Stoff identisch ist mit der orangeroten Verbindung, die unter den *gleichen* Bedingungen gebildet wird und provisorisch als „*Tyrophenazin*" bezeichnet wurde.

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