Studies on Polyphenol Oxidase in Wheat Grains

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ABSTRACT

Polyphenol oxidase was partially purified from the bran fraction of mature grains of tall and dwarf varieties of wheat. The specific activity of the enzyme in dwarfs is distinctly higher than in tall varieties. Further, the enzyme in dwarfs is relatively more thermostable. Substrate specificity studies showed that diphenols are efficient substrates, whereas mono- and polyphenols are poor substrates. Fractionation of isoenzymes of polyphenol oxidase on acrylamide gel electrophoresis revealed three to five bands. Usually the isoenzymes of dwarf varieties are more stable on storage at 4°C. than their counterparts in the tall varieties.

One of the attributes which makes the recently introduced dwarf wheats less acceptable to the consumer is discoloration of whole meal dough and chapatties (unleavened pan-baked bread) (1). Recently, it has been reported that high activity of the enzyme polyphenol oxidase (PPO), localized in the grain coat, is responsible for this discoloration (2). Addition of phenols L-tyrosine, DL-dihydroxyphenyl alanine (dopa), catechol, and chlorogenic acid results in discoloration of chapatties (3).

Studies by Mason (4), Loomis and Battalie (5), Pierpoint (6), and Byck and Dawson (7) have shown that oxidized products of phenols such as quinones may form additional products with the sulfhydryl group of cysteine, ε-amino group of lysine, α-amino group of amino acids and thus affect the properties of the food products or nutritive value of proteins.

In the present communication we report some properties and isoenzyme patterns of partially purified preparations of PPO in some tall and dwarf varieties.

MATERIALS AND METHODS

Wheat grains of two tall varieties (C-591 and K-68) and two dwarf varieties (Sharbati sonora and Sonalika) were selected for the investigation. A Buhler laboratory mill was employed to separate the bran. Catechol (E. Merck), L-tyrosine and phenol (BDH), dopamine, DL-dopa, caffeic acid, and chlorogenic acid (Nutritional Biochemicals) were used as substrates.

Partial Purification of PPO

Twenty grams of bran tissue was homogenized with white sand in 0.05M phosphate buffer (pH 6.0). About 5 ml. buffer per g. bran tissue was used for extraction. The homogenate was passed through a double layer of muslin and centrifuged at 9,000 X g for 10 min. A saturated solution of ammonium sulfate (pH 7.4) was added to the supernatant fraction to give 50% saturation. The precipitated protein was sedimented at 9,000 X g for 10 min. The pellet was dissolved in phosphate buffer (one-fourth its original volume) and dialyzed in cold against phosphate buffer for 16 to 18 hr. The dialyzed fraction was centrifuged at 9,000 X

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g for 10 min. The clear supernatant fraction was treated with calcium phosphate gel (20 mg./ml.) and centrifuged at 5,000 X g to separate the supernatant fraction from the gel. The adsorbed enzyme was eluted batchwise with phosphate buffer (0.4M, 0.6M). The activity of the enzyme was measured in the supernatant as well as in the eluted fractions (Fig. 1).

For separation of isoenzymes of PPO on acrylamide gel electrophoresis the supernatant, as well as elute fractions, was further concentrated by dialysis against solid sucrose.

Assay System for PPO

The incubation mixture contained 1.0 ml. extract (0.14 to 0.40 mg. protein), 1.0 ml. phosphate buffer pH 6 (0.05M), and 2.0 ml. catechol (10 mg./ml.). Activity of the enzyme was measured at 430 nm.

Thermal Stability of Supernatant Fractions

Studies were performed with supernatant fractions. The fractions were maintained at 60°C. for different intervals of time and then chilled in ice. All the fractions were centrifuged at 3,000 X g for 10 min. to remove denatured proteins.

Substrate Specificity

Monophenols (L-tyrosine, 0.18 mg./ml.), diphenols (dopa, catechol, dopamine, caffeic acid, 4 mg./ml.) and polyphenols (chlorogenic acid, 0.02 mg./ml.) were

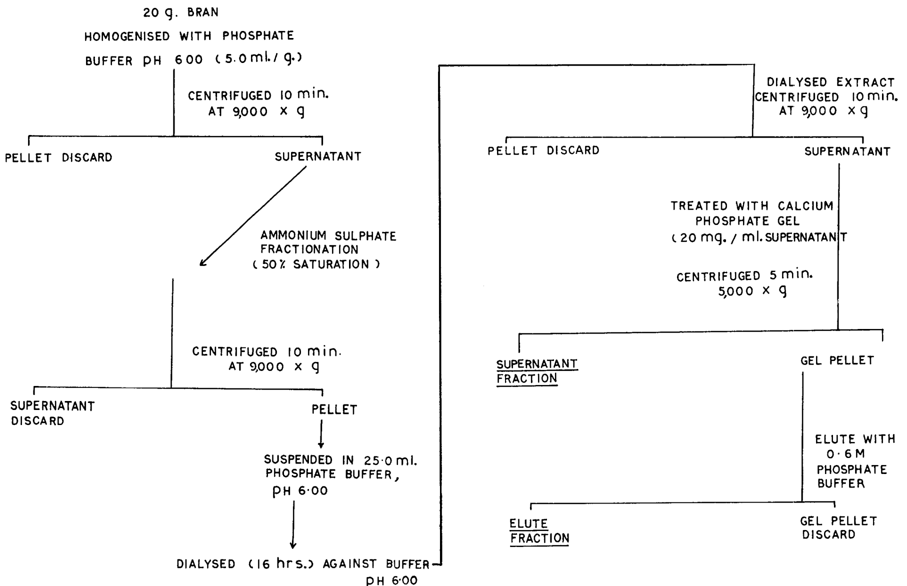


Fig. 1. Procedure followed for the isolation of polyphenol oxidase.

tested for determining the substrate specificity of the enzyme. For monophenols the activity was measured at 280 nm. and for polyphenols at 340 nm.

Isoenzyme Patterns

The method of Davis (8) for acrylamide gel electrophoresis was followed with slight modifications. The gels were polymerized for 45 min. Instead of using spacer gel techniques, the samples were loaded over a 5% sucrose solution. The volume of each sample loaded on each gel column was 0.2 to 0.3 ml. (3 to 4 mg. protein). Substrates like tyrosine, catechol, and dopa were tried for staining the isoenzyme bands. Only dopa in 80% ethanol gave sharp bands. Tyrosine and catechol did not prove effective substrates in gel columns. The procedure of Lowry et al. (9) was followed for estimating proteins in different fractions.

RESULTS

PPO Activity in Tall and Dwarf Varieties

The dwarf varieties invariably showed high specific activity of PPO when compared with the tall varieties. As shown in Figs. 2 and 3, the dwarf variety, Sharbati sonora, had two to three times more activity of the enzyme in supernatant fraction, and about six times more activity in elute fraction.

Substrate Specificity

Various phenolic compounds were tested with tall and dwarf varieties using supernatant and elute fractions. Monophenols, like tyrosine and phenol, did not serve as substrates even after a lag period of 20 to 30 min. The addition of catechol (10 to 100 γ) and dopa (100 γ) in catalytic amounts was also ineffective for the

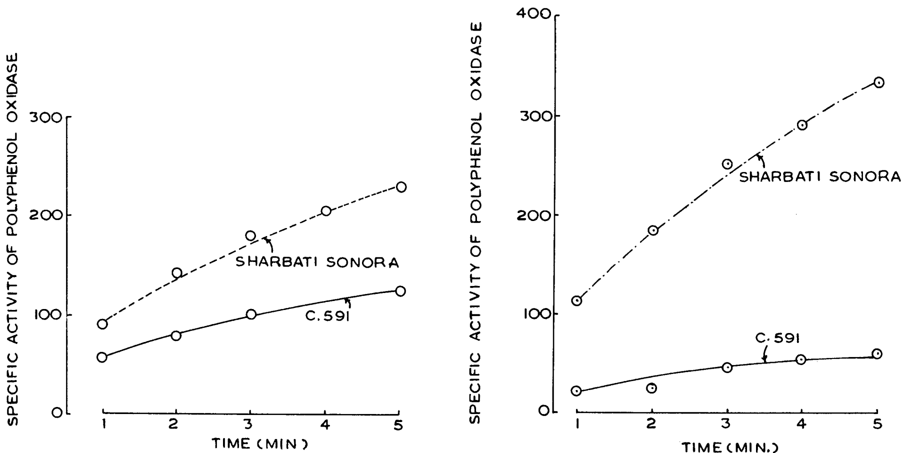


Fig. 2 (left). Comparison of polyphenol oxidase activity in the supernatant fraction of Sharbati sonora and C-591. The incubation mixture contained 1 ml. fraction (2 to 3 mg. protein), 2.0 ml. catechol (10 mg./ml.), and 1.0 ml. phosphate buffer (pH 6.0, 0.05M).

Fig. 3 (right). Comparison of polyphenol oxidase activity in the elute fraction of Sharbati sonora and C-591. The incubation mixture contained 1.0 ml. fraction (2 to 4 mg. protein), 2.0 ml. catechol (10 mg./ml.), and 1.0 ml. of phosphate buffer (pH 6.0, 0.05M).

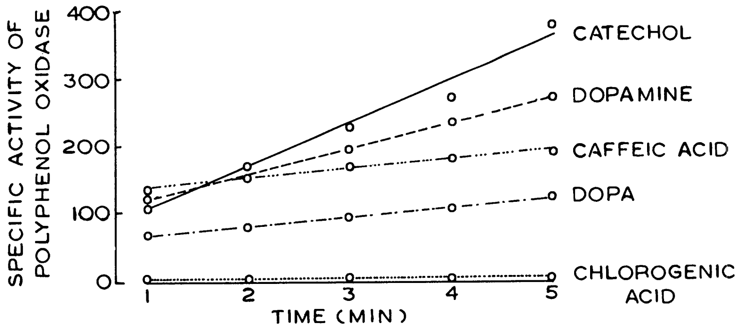


Fig. 4. Kinetics of polyphenol oxidase activity in the supernatant of Sharbati sonora with different substrates indicated in the graph. The incubation mixture contained 2.0 ml. fraction. (3 to 4 mg. protein), 1 ml. of substrate (4 mg./ml.), 1.0 ml. phosphate buffer (pH 6.0, 0.05M). The concentration of chlorogenic acid as substrate was 0.02 mg./ml. The absorbance was taken at 430 nm. for diphenols, and at 340 nm. for chlorogenic acid.

utilization of tyrosine as a substrate. Similarly, the results were negative for chlorogenic acid. Only diphenols proved to be efficient substrates. Their effectiveness varied significantly depending upon the substituent groups on the ring structure. Of the various diphenols tested, catechol proved distinctly superior to other diphenols such as dopamine, caffeic acid, and dopa. The only exception was caffeic acid, which did not serve as a substrate with supernatant fractions of tall varieties and elute fractions of both tall and dwarf varieties (Figs. 4 through 7).

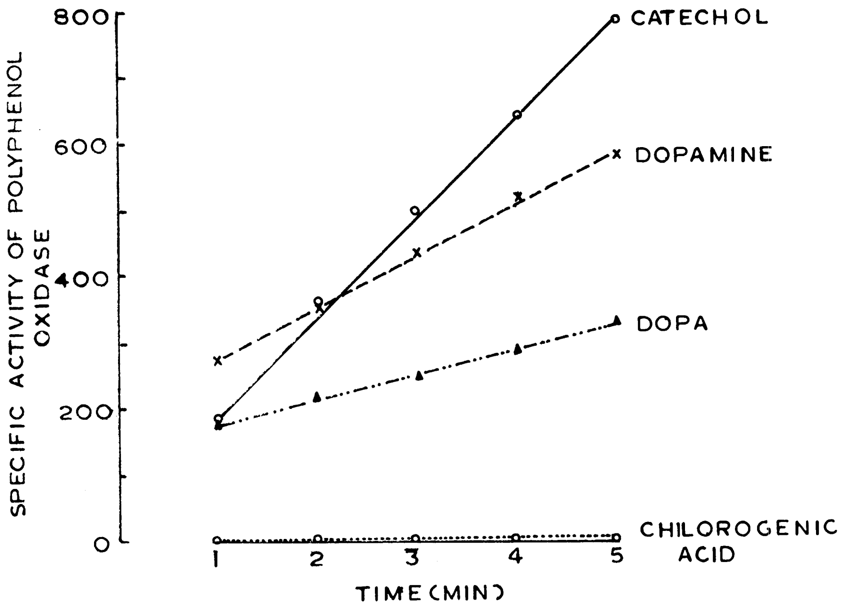


Fig. 5. Kinetics of polyphenol oxidase activity in the elute fraction of Sharbati sonora. Incubation mixture same as in Fig. 4. The elute fraction contained 1.3 mg. protein per ml.

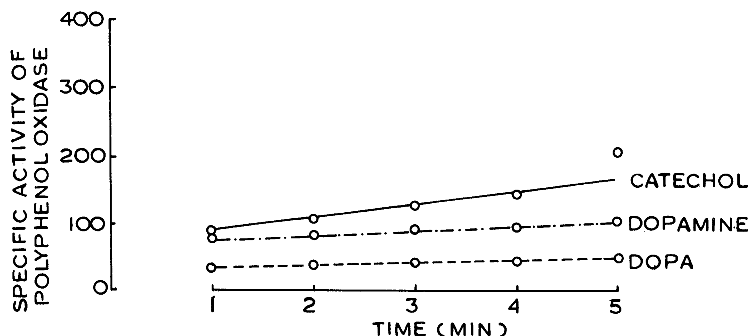


Fig. 6. Kinetics of polyphenol oxidase activity in the supernatant fraction of K-68. Incubation mixture same as in Fig. 4. The supernatant fraction contained 2.6 mg. protein per ml.

Heat Stability

The thermal stability of the enzyme varies significantly in tall and dwarf varieties. The dwarf varieties retained high activity of the enzyme as compared to the tall varieties. Approximately 80 to 85% of the enzyme was thermostable in dwarfs, whereas in the tall varieties the level of activity dropped to 48 to 53% after 1 hr. of heat treatment at 60°C. During the initial phase of heat treatment (10 to 15 min.), loss of activity was significant. Subsequently, the loss was negligible (Figs. 8 and 9).

Previous studies carried out with plant and mushroom PPO have revealed multiple forms of the enzyme (10,11,12). It was considered worthwhile, therefore, to look into the isoenzymatic patterns of PPO in tall and dwarf varieties of wheat.

Patterns of Isoenzymes

In both tall varieties (K-68 and C-591), the supernatant fraction showed three isoenzyme bands. In the dwarf varieties, the supernatant fraction showed three isoenzyme bands for Sonalika and four for Sharbati sonora (Figs. 10 and 11). The elute fractions of Sonalika and K-68 contributed four and five bands, respectively. In order to study the effect of temperature on the thermal stability of isoenzymes,

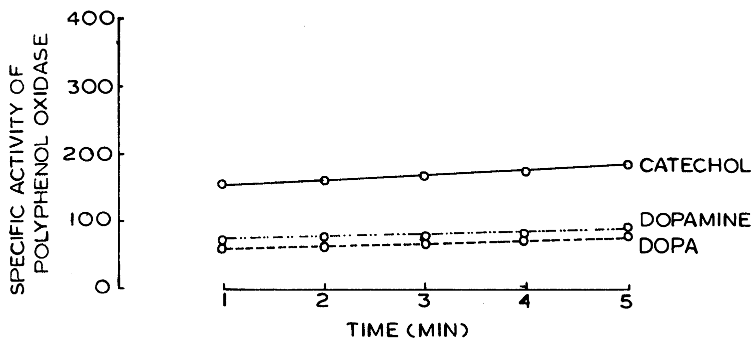


Fig. 7. Kinetics of polyphenol oxidase activity in the elute fraction of K-68. Incubation mixture same as in Fig. 4. The elute fraction contained 1.6 mg. protein per ml.

the supernatant fractions of Sharbati sonora and C-591 were heated at 50°C. for 10 min. After treatment the samples were fractioned on acrylamide gels. The isoenzyme patterns were similar to unheated controls. The stability of isoenzymes was also examined by storing the fractions at 4°C. for 6 days. In dwarf variety Sonalika, the supernatant and elute fractions showed only two bands whereas the fresh preparations showed three to four bands. The disappearance of isoenzymes on storage could be due either to degradation of isoenzymes or its transformation to some more stable form of isoenzyme. In tall varieties like K-68, the isoenzymes in elute fractions seemed to have completely disappeared on storage, and in supernatant fraction all three bands were feebly stained (Fig. 12). However, no general conclusion could be drawn about the stability of isoenzymes in tall and dwarf varieties, since another dwarf variety, Sharbati sonora, also gave feeble bands on storage of supernatant and elute fractions. Further studies are necessary to pinpoint the precise reason for higher stability of isoenzymes in dwarf varieties.

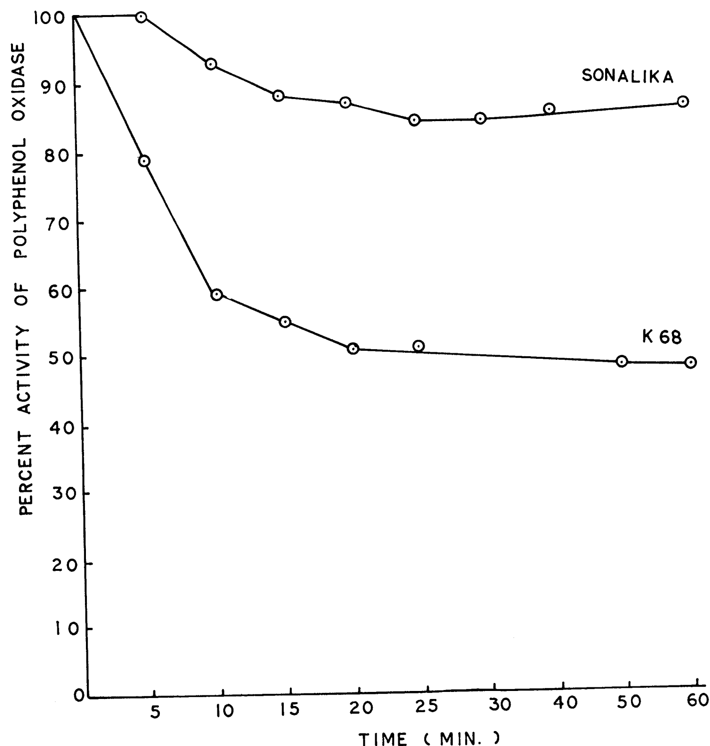


Fig. 8. Heat inactivation curves with the supernatant fractions of Sharbati sonora and C-591. (The activity is expressed per mg. basis of protein measured before heat treatment.) The fractions (C-591, 1.1 mg. protein per ml.; Sharbati sonora, 1.2 mg. protein per ml.) were maintained at 60°C. in water bath for different intervals of time and thereafter chilled on ice. Before measuring the activity of PPO, the fractions were centrifuged at 5,000 × g for 10 min. to remove the denatured proteins. The incubation mixture contained 1.0 ml. supernatant fraction, 1.0 ml. phosphate buffer (pH 6.0; 0.05M), and 2.0 ml. of catechol (10 mg./ml.).

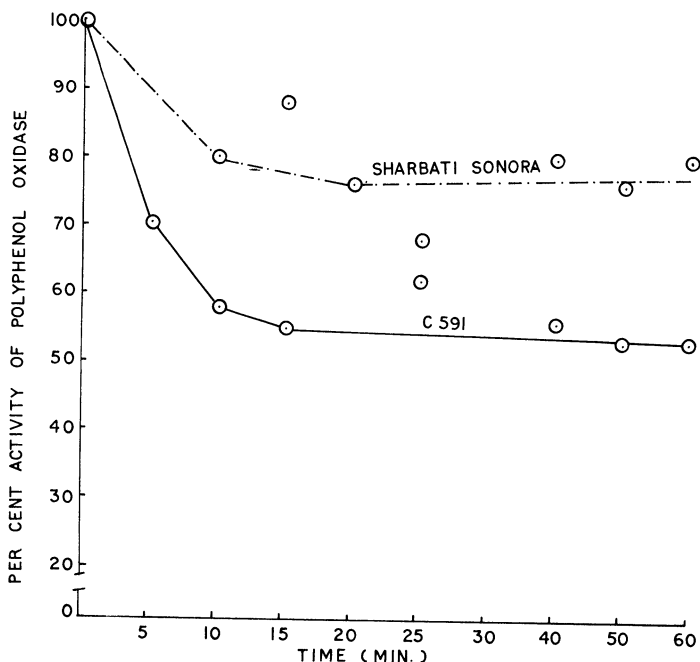


Fig. 9. Heat inactivation curves obtained with the supernatant fractions of Sonalika and K-68. The same experimental procedure was followed as stated in Fig. 8. The fractions contained 1.8 mg./ml. protein.

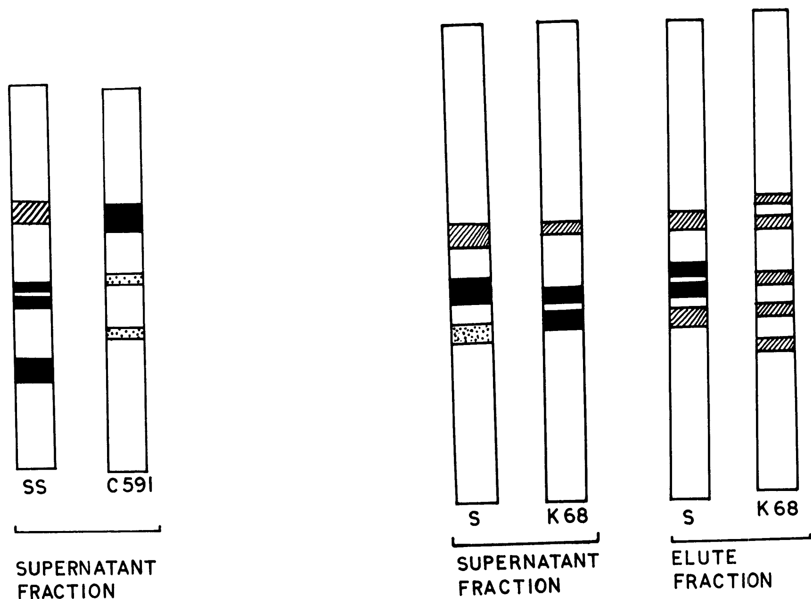
DISCUSSION

Enhancement of oxidative activity in dwarf mutants has been reported by Mathan and Cole (13) in tomato seedlings and by Muller (14) in the case of pea mutants. A comparative study of PPO in tall and dwarf wheats revealed that activity of this enzyme is invariably higher in dwarf wheats (15). Similarly, higher specific activity of PPO in dwarfs was observed in the present study.

Associated with the high enzyme activity is the unusual thermostability of PPO in dwarf varieties. The likelihood of small molecules providing extra stability to PPO of dwarf varieties is excluded in view of elaborate dialysis. These findings may have implications with regard to retention of PPO activity in the chapatties, thereby affecting their discoloration (16).

A study at the isoenzyme level, fractionated on acrylamide gel electrophoresis, failed to show any distinct differences in the number of bands. In general, all the isoenzymes occurring in dwarf varieties were more stable than the ones present in tall varieties. The precise nature of the factor which contributes to the stability of isoenzymes of PPO in dwarf varieties is a subject of further study. Multiple forms of PPO in a number of plant tissues and mushrooms have been reported by various investigators (10,11,12). Earlier attempts to obtain isoenzymes of PPO in wheat grains were unsuccessful (17).

Furthermore, the enzyme PPO could utilize a variety of diphenols, but mono- and polyphenols lacked substrate specificity for this enzyme. Addition of



Figs. 10 (left) and 11 (right). SS, Sharbati sonora; S, Sonalika. Isoenzyme patterns of polyphenol oxidase of supernatant and elute fractions of different varieties of wheat. The samples (0.2 to 0.3 ml.) containing 3 to 4 mg. protein were layered on gel columns containing 5% sucrose. The fractions were run on 7% polyacrylamide gels for 3 to 4 hr. Each column was 3.5 ma. current. The gels were stained by incubating in dopa solution (1.0 mg./ml.) for 3 to 4 hr. The dopa was dissolved in 80% alcohol. The stained gels were stored in 30% alcohol in which the bands persisted for several days.

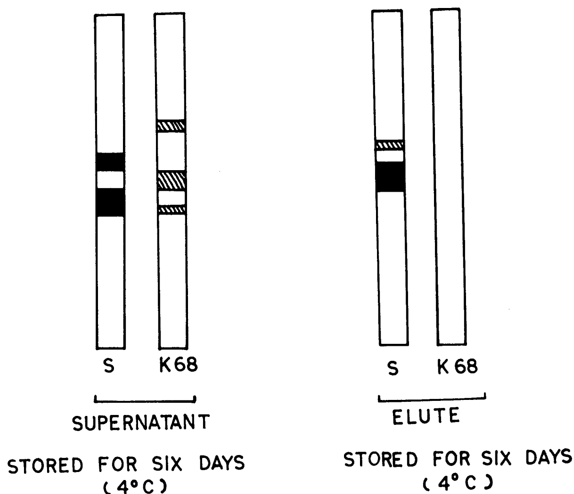


Fig. 12. Gel patterns of polyphenol oxidase after storing the preparations of tall and dwarf varieties for 6 days at 4°C. The supernatant and elute fractions of Sonalika and K-68 were stored for 6 days at 4°C. and then run on acrylamide gels. The gels were stained in dopa prepared in 80% ethanol.

L-tyrosine and chlorogenic acid into whole meal results in discoloration of whole meal dough and chapatties (3). This raises the possibility of certain phenols (which do not serve as substrates of PPO) interacting with quinones, and thus resulting in formation of colored products (18).

PPO from other sources, such as mushrooms, is quite effective against monophenols and polyphenols (11,12). During the present investigations also it was observed that L-tyrosine can be utilized by PPO if the wheat seeds are soaked overnight. This opens up the possibility of the occurrence of new multiple forms of isoenzymes during seed germination. The new activity which can accept monophenols could be new synthesis, or a case of modification of the existing enzyme. Further work is in progress to settle this issue. It will also be interesting to look into the utilization of chlorogenic acid by soaked seeds.

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