Modulation of o-Diphenolase and Monophenolase Enzymes during Wheat Grain Development

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ABSTRACT

The activity and isoenzymes of polyphenol oxidase have been examined in the developing grains of wheat. There was no synchrony in the appearance of monophenolase and *o*-diphenolase activities during the development of seed. The *o*-diphenolase activity is considerably high in young seeds, while the monophenolase activity is practically undetectable throughout the major period of grain filling. The induction of monophenolase activity occurs with the onset of grain ripening, while at this stage, a 60-fold reduction was witnessed in *o*-diphenolase activity. Further, the developing grains revealed 9 to 10 isoenzymes of *o*-diphenolase activity and only a single activity band of monophenolase.

Interest in the enzyme polyphenol oxidase (PPO) arises from its role in phenolic biosynthesis (1,2), broad substrate specificity, existence in multiple forms (3,4,5), and oxidation of phenols *vis-a-vis* formation of dark-colored pigment in biological materials and food products (6,7). Incidence of high PPO activity in dwarf wheats (8,9) and high oxidase activity as a possible biochemical basis for dwarfness (10,11) arouses further interest in this enzyme especially in view of the recent introduction of high-yielding dwarf wheats. High activity of the PPO enzyme in the wheat grains of dwarf varieties was reported to be the causative factor for discoloration of whole meal and chapatties (12, 13).

Our earlier studies showed that this enzyme is relatively more thermostable in dwarf varieties than the tall ones. Diphenols serve as more efficient substrates than mono- and polyphenols (14). Three to five isoenzymes of PPO activity with DL-dopa were observed (9).

In this communication, studies on the activity and isoenzymes pattern of PPO of two wheat (*Triticum aestivum*) varieties, namely Shabati Sonora, a dwarf variety, and NP 875, a tall variety but with high PPO activity, are reported.

MATERIALS AND METHODS

Material

Wheat varieties, Sharbati Sonora and NP 875, were grown on the farms of the Indian Agricultural Research Institute. The spikes of mother shoots were tagged on the day of anthesis. The developing grains were excised at a regular interval of 3 days till the seeds attained maturity.

Enzyme Extraction

Four grams material (including seed coat) was homogenized with 12.0 ml. of chilled phosphate buffer (0.05M, pH 6.6) and centrifuged in Sorvall at 20,000 × g for 15 min. at 4°C. The supernatant fraction (crude extract) was stored in cold and used for the assay of polyphenol oxidase.

Enzyme Assay

The crude extract was tested for the activity of polyphenol oxidase using catechol (10.0 mg. per ml.) and L-tyrosine (1.0 mg. per ml.) as substrates. The incubation mixture contained 2.0 ml. of catechol, 0.1 ml. of crude extract, and phosphate buffer (0.05M, pH 6.6) added to make the final volume of 4.0 ml. Prior to the addition of enzyme, the mixture of catechol and phosphate buffer was incubated at 37°C. for 2 min. The absorbancy was measured at intervals of 15 sec. at 430 nm. The monophenolase activity was assayed with 2.0 ml. L-tyrosine (1.0 mg. per ml.) solution and 0.5 ml. of crude extract together with 1.5 ml. of phosphate buffer. Prior to the addition of crude extract, the substrate was oxygenated for 5 min. The absorbancy was taken at 430 nm. after 4 hr. of incubation at 37°C. Omission of substrates from the incubation mixture served as controls.

Acrylamide Gel Eiectrophoresis

The crude extract was fractionated on polyacrylamide gel (7.5%) electrophoresis (9,15) using *tris*-glycine buffer (pH 8.3) for location of monophenolase and *o*-diphenolase activities. About 1,800 to 2,000 γ protein sample was layered on

each gel column. The gels were stained with DL-dopa (1.5 mg./ml.) and L-tyrosine (1.0 mg. per ml.) solutions prepared in 80% ethanol, destained, and stored in 30% alcohol (4). To eliminate the possibility of exogenously added phenolics (DL-dopa, L-tyrosine) reacting with peroxidase isoenzymes in the unwashed gels, the enzyme extracts were also fractionated on electrophoretically prewashed acrylamide gels (16). Since unwashed and exhaustively washed gels showed no difference in the banding pattern of monophenolase and *o*-diphenolase isoenzymes, subsequent experiments were performed only with unwashed gels.

Protein Assay

Protein was assayed according to the procedure of Lowry et al. (17).

RESULTS

o-Diphenolase Activity

The *o*-diphenolase activity has been traced in the developing grains of wheat, beginning from the day of anthesis till the seeds attained maturity in 48 days. The early stages of grain development revealed a very high activity of *o*-diphenolase. This became evident when the enzyme activity of young seeds was compared with the seeds at maturation phase. The extracts, prepared from 9-day-old seeds, revealed a 60-fold higher *o*-diphenolase activity than the ripe seeds. As shown in Fig. 1, the pattern of *o*-diphenolase activity in developing grains of Sharbati Sonora

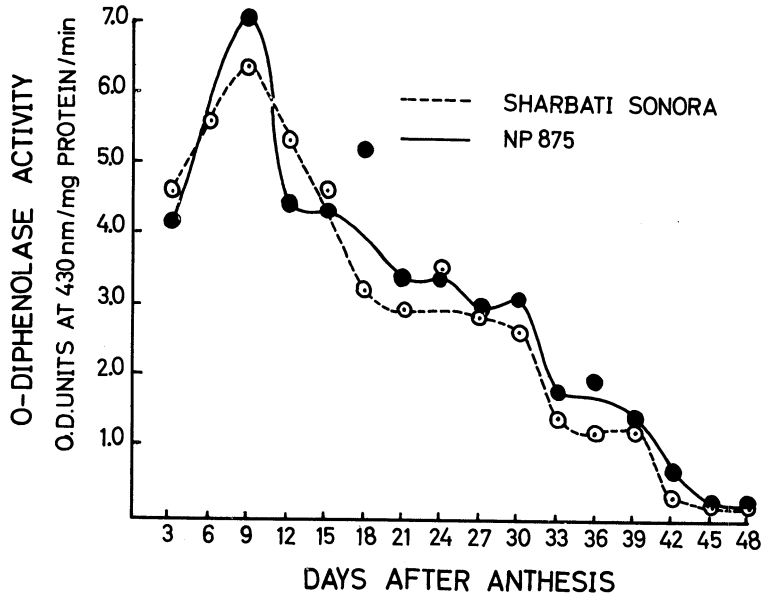

Fig. 1. *o*-Diphenolase activity in the developing grains of two varieties of wheat. The incubation mixture contained 0.1 ml. of crude extract, 2.0 ml. of catechol (10.0 mg. per ml.) and phosphate buffer (0.05M, pH 6.6) to make the final volume 4.0 ml. Omission of substrate served as control. The reaction was carried out for 1 min. at 37°C. and absorbancy measured at 430 nm.

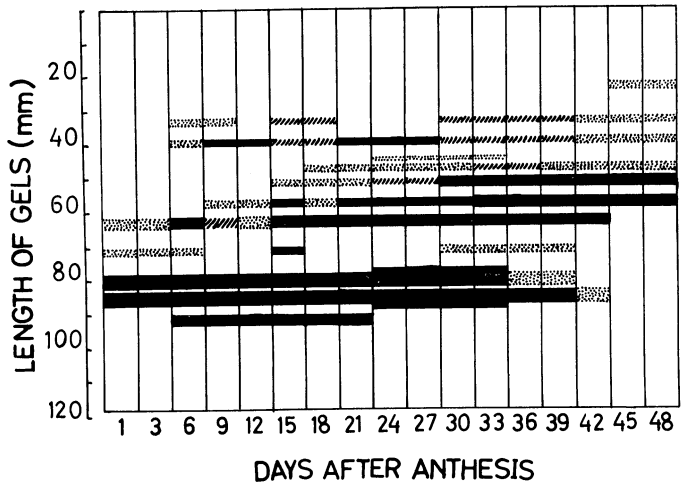

Fig. 2. Diagrammatic representation of *o*-diphenolase isoenzymes in wheat (var. Sharbati sonora) at different stages of grain development. The crude extract was fractionated on polyacrylamide gels (7.5%) using *tris*-glycine buffer (pH 8.3). The sample (1,800 to 2,000 γ protein) containing 10% sucrose solution was layered over 5% sucrose solution on each gel column. Bromophenol was used as a tracking dye in the system. The isoenzyme bands were developed by incubating the gels in DL-dopa (1.5 mg. per ml., prepared in 80% ethanol) at 37° C. and subsequently destained and stored in 30% ethanol. ▪, High activity; ▨, medium and ⁘, low activity.

and NP 875 appeared to be identical. With increasing age of the grain there was a gradual decrease in the activity of *o*-diphenolase.

Isoenzymes of *o*-Diphenolase

The pattern of isoenzymes of *o*-diphenolase activity varied considerably during the course of grain development. At anthesis, the extract prepared from the ovaries showed four iosenzymes of *o*-diphenolase activity, two of which were fast-moving and gave intense activity with DL-dopa (Fig. 2). There was no change in the number of isoenzymes at three days after anthesis. In 6-day-old seeds, a single fast-moving and two slow-moving bands were added to the existing isoenzymes. At 24 days after anthesis, the fastest-moving band vanished while the adjacent two prominent fast-moving bands presisted in a 33-day-old grain. Maximum number of 10 multiple forms was observed at 30-to-33-day stage. With the onset of maturation of grain (i.e., 36 days after anthesis), one of the fast-moving bands gave weak activity, and subsequently both of them were no longer detectable in 45-day-old seed. In addition, a further decline in the number of isoenzymes was observed and in fully developed seeds, there were only six isoenzymes of *o*-diphenolase activity. Briefly, then, the fast-moving isoenzymes present in the early stages of development disappear with the onset of maturation (Fig. 2). The general pattern of *o*-diphenolase isoenzymes was essentially identical in varieties Sharbati Sonora and NP 875 (Figs. 3a and 3b).

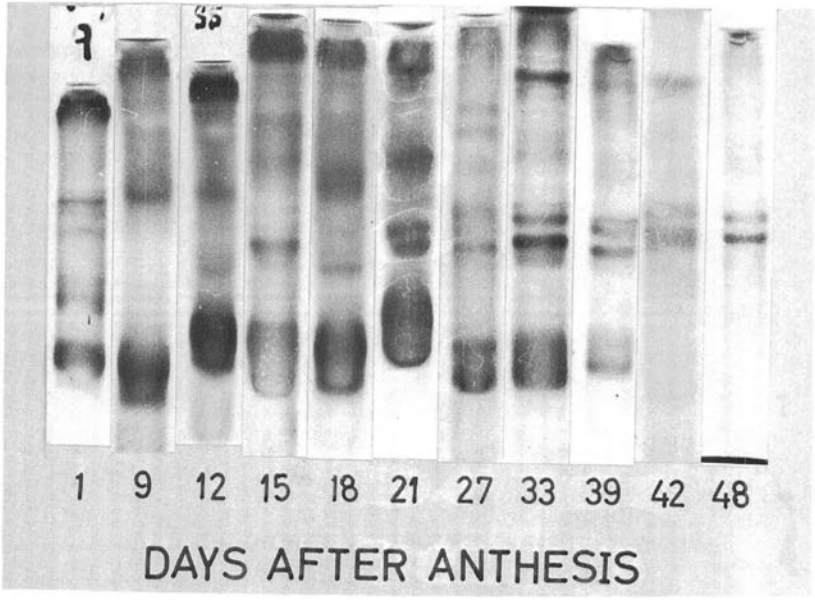

Fig. 3a. Photomicrographs of acrylamide gels showing electrophoretic patterns of *o*-diphenolase isoenzymes in wheat (var. Shabati Sonora). For details of procedure see legend of Fig. 2.

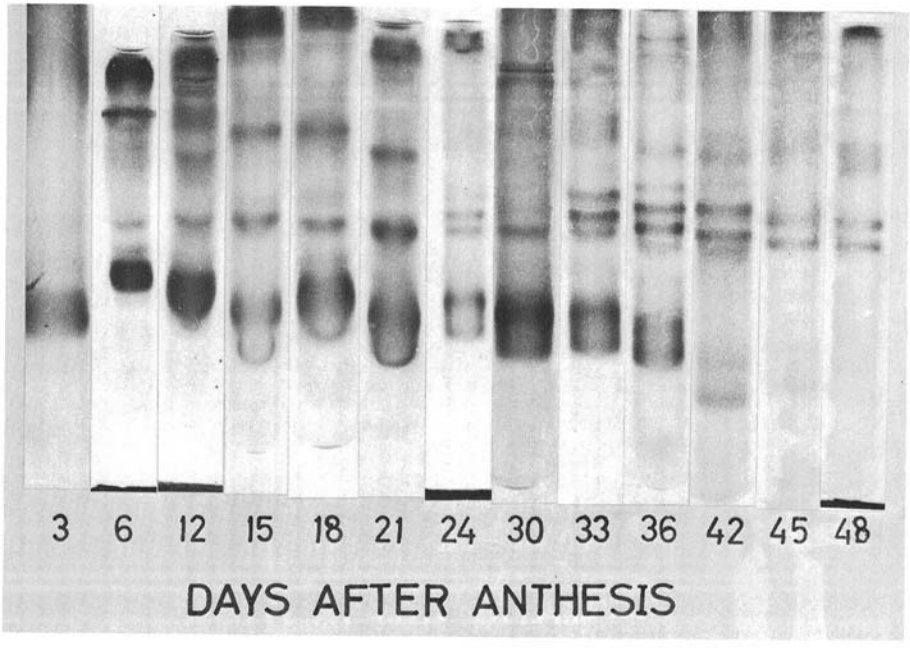

Fig. 3b. Photomicrographs of acrylamide gels showing electrophoretic pattern of *o*-diphenolase isoenzymes in wheat (var. NP 875). For details of procedure see legend of Fig. 2.

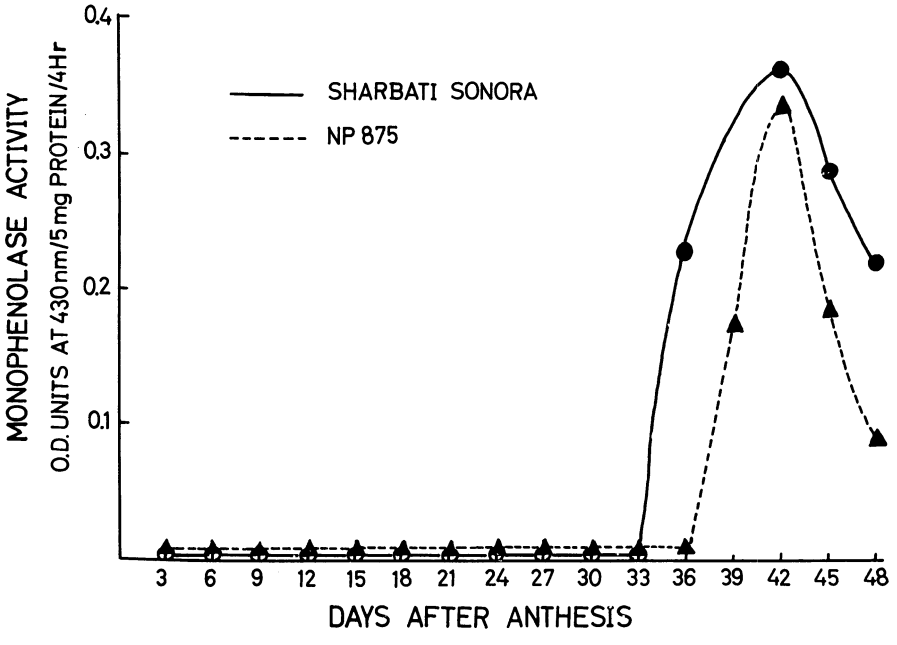

Fig. 4. Monophenolase activity in developing grains of two varieties of wheat. The incubation mixture contained 0.5 ml. of crude extract, 2.0 ml. of L-tyrosine (1.0 mg. per ml.), and 1.5 ml. of 0.05M phosphate buffer (pH 6.6). The assay mixture without the enzyme was oxygenated for a period of 5 min. Omission of substrate served as control. The absorbancy was measured at 430 nm. after the incubation mixture was incubated for 4 hr. at 37°C.

Activity and Gel Pattern of Monophenolase

In contrast to *o*-diphenolase activity which was remarkably high in the early stages of grain development, the monophenolase activity was not detectable until the seeds approached the ripening phase (36 to 39 days). Maximum monophenolase activity appeared only at 42 days after anthesis. In mature seeds, the activity of this enzyme was low in NP 875 as compared to Sharbati Sonora (Fig. 4).

The extracts, prepared from seeds of different stages, were fractionated on gel electrophoresis for examining the isoenzymes of monophenolase. No activity band of monophenolase was observed on gels for the first 33 days of grain development. A single band of monophenolase activity appeared at 36 days after anthesis (Fig. 5). It became more intense up to 42 days, but eventually decreased in intensity in fully mature seeds (48 days). Unlike *o*-diphenolase, no multiple forms were observed for monophenolase activity. The time of appearance of monophenolase activity in NP 875 and Sharbati Sonora was identical. The appearance of L-tyrosine specific activity (monophenolase) band appears to be linked with the onset of maturation phase wherein the seed starts turning brown from its initial green color, indicating that monophenolase may have a possible role in the formation of pigments in grain coat.

DISCUSSION

The present findings revealed that *o*-diphenolase and monophenolase activities

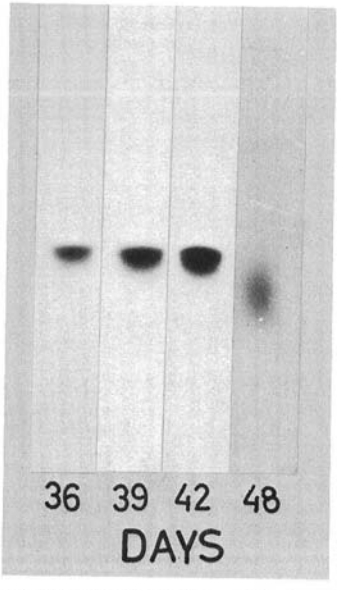

Fig. 5. Photomicrographs of acrylamide gels showing electrophoretic pattern of monophenolase activity band in wheat (var. Sharbati Sonora) at different stages of grain development. The crude extract was fractionated on acrylamide gels (7.5%) using *tris*-glycine buffer (pH 8.3). The sample (1,800 to 2,000 γ protein) containing 10% sucrose solution was layered over 5% sucrose solution on each gel column. The monophenolase activity band was developed by incubating the gels in L-tyrosine (1.0 mg. per ml. in 80% ethanol) solution at 37°C. The gels were destained in 30% alcohol.

follow distinctly different patterns during grain development. During the early stages of grain development the activity of *o*-diphenolase was very high while there was no trace of monophenolase activity at this stage. In mature grains, the *o*-diphenolase activity dropped considerably whereas the monophenolase showed highest activity. Nevertheless, the activity of *o*-diphenolase is considerably higher than the monophenolase activity in mature grains. The lack of synchrony in the appearance of these two activities during grain development suggests that monophenolase and *o*-diphenolase represent two distinct enzymes and not a part of the same enzyme complex. Further evidence in support of this hypothesis has been provided in another communication.[1]

In our earlier study, little or no monophenolase activity was detectable in the mature wheat grains (9). It is quite likely that partial purification of the enzyme resulted in the loss of monophenolase activity as reported by a number of investigators (18,19). Wong et al. (5), however, did not observe any monophenolase activity in crude homogenates of clingstone peach.

Multiple forms of *o*-diphenolase have been reported in a number of tissues (4,5,9). Wong et al. (5) reported differences in the properties of various isoenzymes, namely, optimal pH, heat stability, half-life, etc. Multiple forms of *o*-diphenolase

[1]Taneja, S. R., and Sachar, R. C. Localization of monophenolase and *o*-diphenolase activities of polyphenol oxidase on separate enzymes in wheat (accepted in Phytochemistry, 1974).

are known to occur by association of monomeric units (20). The slow-moving bands of wheat get converted to fast-moving band when the extracts prepared from embryo, excised from 2-day-old seedlings, were treated with sodium dodecyl sulfate (0.1%) and mercaptoethanol (0.1%) (21). In the present study, 9 to 10 isoenzyme bands of *o*-diphenolase activity were observed. Initially there were 4 to 5 fast-moving bands and these increased to 9 to 10 at the 30- to 33-day stage of development. In mature grains six slow-moving bands were detectable. It is quite likely that this shift is brought about by the association of monomers to polymeric forms.

So far, phenol color reaction with monophenols has been employed as an index of PPO activity in wheat. However, the present studies have revealed that the activity of *o*-diphenolase is distinctly higher than the monophenolase activity in mature seeds. Moreover, the recent studies have shown that *o*-diphenolase activity is present in the embryo and the grain coat tissue, while the monophenolase activity is restricted to the grain coat (21). In view of these findings, the use of diphenols may provide a more sensitive test for determining PPO activity in wheat grains.

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