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SEPARATE MONOPHENOLASE AND *o*-DIPHENOLASE ENZYMES IN *TRITICUM AESTIVUM*

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Abstract—Monophenolase and *o*-diphenolase activities of polyphenol oxidase are usually thought to be a part of the same enzyme complex It has now been demonstrated that the two catalytic activities of the polyphenol oxidase of wheat grains are separable and reside in different enzymes The electrophoretically separated monophenolase enzyme showed specificity only for monophenol (L-tyrosine) after its elution from acrylamide gels Further, this enzyme is confined to the endosperm tissue and is undetectable in the embryonic region of the seedling

INTRODUCTION

POLYPHENOL oxidase, commonly referred to as tyrosinase, is of wide occurrence in plants, animals and micro-organisms. This enzyme catalyzes the oxidation of mono, di- and polyhydric phenols. Previously it was reported that the monophenolase (cresolase) and *o*-diphenolase (catecholase) activities are located on the same enzyme molecule. The monophenolase activity is less stable than the *o*-diphenolase activity.[1] Depending upon the procedure of purification, this enzyme can be obtained with high monophenolase or *o*-diphenolase activity. Polyphenol oxidases isolated from peach,[2] tea,[3] tobacco,[4] banana[5] and *Prunus*[6] have only *o*-diphenolase activity. In animal system, a pteridine dependent tyrosine hydroxylase (monophenolase) is reported, in addition to the existence of tyrosinase with mono and diphenol functions.[7] In *Sorghum*, three phenol oxidase activities have been separated by Sephadex G100 gel filtration. A high MW form has both monophenol and diphenol oxidase functions; an intermediate MW form exhibits only a diphenol function; while a low MW form shows monophenol activity and may possess weak diphenol activity.[8] However, the classical polyphenol oxidase is considered to be a single complex having independent sites for monophenol and diphenol functions.[9–11] In this communication, we report the electrophoretic separation of monophenolase and *o*-diphenolase activities of wheat polyphenol oxidase which are associated with different enzymes.

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[10] MASON, H S (1957) *Adv Enzymol* **19,** 79
[11] MATHEW, A G and PARPIA, H A B (1971) *Adv Food Res* **19,** 75

RESULTS AND DISCUSSION

Polyphenol oxidase, isolated from mature grains of wheat, was tested for activity with mono- and diphenols as substrates (Table 1) All 3 fractions showed high *o*-diphenolase activity with catechol, whereas the monophenolase activity was extremely weak when tested with L-tyrosine. Since this enzyme is known to exist in multiple forms,[12,13] we have separated the isoenzymes of polyphenol oxidase on acrylamide gels and studied their relative substrate specificity for monophenols and diphenols. In crude extracts, the polyphenol oxidase showed 6 bands of isoenzymes after incubating the gels with DL-dopa and a single broad band with L-tyrosine. No bands were visible on gels incubated with other monophenols such as phenol, *p*-cresol and vanillic acid. Therefore, for all subsequent experiments L-tyrosine was employed for staining the gels for monophenolase activity The band obtained with L-tyrosine in crude extracts was a single fast moving band which did not overlap with any of the six bands of *o*-diphenolase activity. This indicated that the catecholase and cresolase activities may be associated with different multiple forms. Since a feeble band of monophenolase activity appeared with L-tyrosine, attempts were made to obtain enriched fractions of polyphenol oxidase. Ammonium sulphate fractionation together with precipitation with ethanol were employed for concentrating the activity of the preparation (Table 1). Further studies were confined to alcohol *Fractions I* and *II* The gel electrophoretic pattern of *Fraction II* revealed 7 bands of *o*-diphenolase activity and two of monophenolase activity when 2 separate gels were incubated with DL-dopa and L-tyrosine respectively. However, it is significant to note that out of the 2 bands obtained with L-tyrosine, one was in a position which coincided with some of the bands obtained with diphenols, while the other was characteristic in occupying a position on the gel where no *o*-diphenolase activity was observed in the corresponding gel. Thus there could be a possibility of the existence of isoenzymes which carry either monophenolase or *o*-diphenolase activity. This view has been further elucidated on examining the electrophoretic pattern of polyphenol oxidase in *Fraction I*. In this Fraction, it has been possible to achieve the complete separation of *o*-diphenolase from monophenolase activity Four bands of *o*-diphenolase activity were observed when the gels were incubated in DL-dopa and a single band of monophenolase activity appeared with L-tyrosine Comparison of the gels of *Fraction I* clearly showed that the monophenolase activity appeared in a position distinct from the isoenzyme bands of *o*-diphenolase activity. These studies indicated that the catalytic sites of *o*-diphenolase and monophenolase do not reside in the same enzyme complex in *Fraction I* Since previous workers have considered cresolase and catecholase activities to be part of the same enzyme complex,[9,13] further characterization was necessary to confirm our findings regarding the presence of two activities on separate enzymes. The fast moving monophenolase band was therefore eluted from the unstained gels of *Fraction II*, re-fractionated on acrylamide gels and subsequently tested with mono- and diphenols No change was observed in the position of this band on gels; it retained specificity for L-tyrosine and showed no activity with DL-dopa. Also the eluted fraction when tested colorimetrically, showed specificity for L-tyrosine as a substrate and did not respond to diphenols like DL-dopa or catechol We, therefore, propose that the monophenolase activity is physically separable from *o*-diphenolase activity and does not undergo any association or dissociation following its elution from the acrylamide gels. Further, we do not believe that the fast moving monophenolase activity has lost its site for *o*-diphenolase activity during puri-

[12] CONSTANTINIDES, S M and BLDFORD, C L (1967) *J Food Sci* **32**, 446
[13] IOLLEY, R L JR., ROBB, D A, and MASON, H S (1969) *J Biol Chem* **244**, 1593

fication steps, since this activity was also observed in a feeble form in our crude extract. Similarly isoenzymes showing o-diphenolase activity failed to give any reaction with L-tyrosine even after prolonged incubation.

The above studies on polyphenol oxidase were confined to the mature grains of wheat, where monophenolase and o-diphenolase activities reside in separate enzyme molecules. One might argue that one of the catalytic sites in different isoenzymes becomes inactivated during the maturation of the seeds This possibility was ruled out after examining monophenolase and o-diphenolase activities in the developing grains of wheat The study of polyphenol oxidase in the early stages of grain development revealed no detectable band of monophenolase All the isoenzymes exhibited o-diphenolase activity. However, the monophenolase activity appeared in the later stages of maturing grains and is physically separable from o-diphenolase activity.[14] Similarly, there was no indication of the presence of both catalytic sites on the same enzyme during the germination of seed. The excised coleoptile-root portion of 2-day-old seedling showed only o-diphenolase activity. On the other hand, the embryo-less half seeds, dissected from the seedlings, showed both monophenolase and o-diphenolase activities which could be separated electrophoretically. The o-diphenolase isoenzymes were slow moving as compared to the fast-moving monophenolase activity. Thus, the developmental and germination studies of the grains lend further support to our view that the two activities of polyphenol oxidase represent two distinct enzymes in wheat. The similarity in the absorption spectrum of the end-product of L-tyrosine and DL-dopa clearly indicated that the melanin pigment formed with mono- and diphenols is identical.

TABLE 1 POLYPHENOL OXIDASE ACTIVITY IN DIFFERENT FRACTIONS OF WHEAT GRAINS WITH CATECHOL AND L-TYROSINE

Substrate	Time	Absorbance units at 430 nm/5 mg protein		
		Crude extract	*Fraction I*	*Fraction II*
Catechol	5 min	0 45	1 7	2 0
L-tyrosine	4 hr	0 25	0 4	0 5

The incubation mixture contained 1 ml of enzyme fraction, 2 ml of substrate solution and 1 ml of 0 05 M phosphate buffer (pH 6 6) Catechol (10 mg/ml) and L-tyrosine (0 5 mg/ml) solutions were prepared in phosphate buffer (0 05 M, pH 6 6) Omission of substrate from the reaction mixture served as control

Since it is reported that the endogenous peroxides produced by ammonium persulphate are present in unwashed acrylamide gels,[15] it was necessary to determine the possible participation of peroxidase activity in gels incubated with L-tyrosine and DL-dopa. In order to eliminate the presence of peroxides, electrophoretically washed gels were employed for the run. Such gels after staining with L-tyrosine showed a single band which was identical to the one obtained with unwashed gels. Thus, the band obtained with L-tyrosine in unwashed, as well as prewashed gels, could only represent a monophenolase catalyzed reaction. Similarly, there was no change in the pattern and number of o-diphenolase isoenzymes when the unwashed and prewashed gels were stained with DL-dopa. The reason for not finding any contamination of peroxides in unwashed gels could be explained on the basis that normally penetration and concentration of the enzyme fraction into the gels took nearly 60 min and after flushing the gels, the electrophoretic run was completed in

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[15] SHEEN, S J (1972) *Evolution* **26**, 143

another 60 min. It is therefore very likely that the traces of peroxides are removed from unwashed gels during this time and that exhaustive pre-washing of the gels is unnecessary for our system. In order to further substantiate this view point, the unwashed gels were stained with benzidine without the exogenous addition of hydrogen peroxide. No peroxidase isoenzyme bands appeared on the gels. Similarly, benzidine added to the unwashed gels, previously incubated with L-tyrosine solution prepared in 80% ethanol, did not show any augmentation in the number of bands. On the other hand, the addition of hydrogen peroxide (1%), together with benzidine, was necessary to obtain the isoenzyme bands of peroxidase activity. Clearly therefore, no peroxidase isoenzymes would develop on unwashed gels with the substrate benzidine unless hydrogen peroxide is also added to the incubation mixture. Thus the possibility of phenolics reacting with peroxidase bands due to contamination of unwashed gels with peroxides is untenable, since the exhaustively washed gels also showed activity band with L-tyrosine. Obviously, therefore, peroxidase isoenzymes cannot react with L-tyrosine for want of hydrogen peroxide in our system and the appearance of fast moving band with this monophenol (L-tyrosine) represents a monophenolase catalyzed reaction.

EXPERIMENTAL

Extraction and preparation of fractions. Polyphenol oxidase was extracted from powdered wheat (100 g) gr (*Triticum aestivum*, c v *Sharbati Sonora*) with 300 ml 0·05 M phosphate buffer (pH 6·6). The homogenate was centrifuged for 10 min at 13 000 *g*. The supernatant (*Crude Extract*) was precipitated with 60% saturated $(NH_4)_2SO_4$. The ppt was suspended in 50 ml 0·05 M phosphate buffer and the salt removed by passing the $(NH_4)_2SO_4$ fraction through Sephadex G25. The G25 eluate was precipitated with equal vols of EtOH at −5° and centrifuged at 16 000 *g* for 20 min. The supernatant was retained for further fractionation. The pellet was air dried to remove traces of EtOH, suspended in 10 ml of phosphate buffer and centrifuged at 20 000 *g* for 1 hr to obtain a clear soln. This was designated as *Fraction I*. The supernatant obtained after the EtOH step was further fractionated with same vol of EtOH as added previously. The ppt was suspended in phosphate buffer (10 ml) and is designated *Fraction II*.

Enzyme assays. The activity of polyphenol oxidase was measured colorimetrically at 430 nm using catechol, DL-dopa and L-tyrosine as substrates. The incubation mixture contained 1 ml of the enzyme fraction, 2 ml of substrate soln and 1 ml of 0·05 M phosphate buffer (pH 6·6). The concentration of catechol and L-tyrosine were 10 mg/ml and 0·5 mg/ml respectively. The substrate and the enzyme fraction were mixed after a pre-incubation period of 2 min at 37°. Omission of substrate from the assay mixture served as control. The increased absorbance due to enzyme activity is expressed per mg of protein. For assay of monophenolase activity the reaction mixture containing L-tyrosine was oxygenated for 5 min prior to the addition of enzyme extract.

Polyacrylamide gel electrophoresis. The *Crude Extract* and *Fractions I, II* were run on polyacrylamide gel (7·5%) electrophoresis using Tris–glycine buffer (pH 8·3).[16] The gels were also exhaustively washed electrophoretically for a period of 6 hr before loading the samples on the gels.[15] The *Crude Extract*, prepared from seedlings and the developing grains of wheat were also run on gel electrophoresis and tested with L-tyrosine and DL-dopa. Approximately 1·5 mg of protein sample in 10% sucrose was layered over 5% sucrose soln on each gel column. The gels were stained in DL-dopa (1·5 mg/ml), L-tyrosine (0·5 mg/ml), phenol (1 mg/ml), *p*-cresol (1 mg ml) and vanillic acid (1 mg/ml) solns prepared in 80% EtOH and incubated at 37° for developing the isoenzyme bands of polyphenol oxidase. The gels were destained and stored in 30% EtOH.[12] The fast moving cresolase activity from unstained gels of *Fraction II* was eluted with phosphate buffer (0·05 M, pH 6·6) and re-fractionated on fresh gel columns for testing its activity with mono- and diphenols. The preparation was also assayed colorimetrically. The eluted fraction was extracted from unstained gels of fraction II from a region which corresponded to fast moving monophenolase activity. The gel segments (5 mm in length) of this region were excised from six gels minced with a glass rod and stirred constantly with 3 ml of phosphate buffer (0·05 M, pH 6·6) for 30 min. The gel was removed by centrifugation and the supernatant served as eluted fraction. The extracts of blank gel together with different substrates served as controls. Each incubation mixture contained 0·7 ml of enzyme extract from the eluted fraction and was tested with 0·7 ml of L-tyrosine (0·5 mg/ml in 80% EtOH), DL-dopa (2 mg/ml in 80% EtOH) and catechol (2 mg/ml in 0·05 M phosphate buffer) solns respectively. The assay mixture was incubated for 4 hr at 37°. For ascertaining the role of endogenous peroxides produced from ammonium persulphate in the unwashed acrylamide gels, the same were stained with a soln of benzidine alone, and also with equal vols

[16] DAVIS, B J (1964) *Ann N Y Acad Sci* **121**, 265

of benzidine and 1% H_2O_2 and subsequently cleared in 7% HOAc A satd soln of benzidine was prepared in 25% HOAc Benzidine soln was also added to unwashed gels incubated with L-tyrosine

Protein assay The *Crude Extract* was precipitated with equal vols of cold 10% CCl_3 COOH The pellet was suspended in 0 3 M KOH solution and the protein assayed by Lowry's procedure[17] using bovine serum albumin as the standard

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