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CHANGES IN THE ACTIVITIES OF SEVERAL ENZYMES DURING GERMINATION AND SEEDLING DEVELOPMENT IN CORN (ZEA MAYS L.) [1,2,3]

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The literature contains numerous references (e.g., Bonner (1), Van Fleet (18), Weier and Stocking (19)) to the occurrence of catalase, cytochrome oxidase, peroxidase, phosphatase, and polyphenolase activities in higher plants, and an occasional report has appeared dealing with certain of these activities in corn. Thus there have been reports of cytochrome oxidase (4, 9), peroxidase (2), catalase (2, 3, 13), and phosphatase (8) activities in preparations of various corn tissues. In most cases, however, only one stage of development has been considered. The experiments reported here were undertaken when, in connection with other studies in progress at this laboratory, it became necessary to obtain estimates of the five types of activity in preparations of corn embryos, etiolated shoots, and green seedlings. The aim of these experiments was threefold: to adapt published assay methods for use with preparations of corn tissue, to estimate the activities of preparations from corn at the various stages of development listed above, and to study the fractionation of activities effected by high-speed centrifugation.

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Materials and Methods

Seeds of corn (*Zea mays* L.) of the variety, Krug, and the hybrid, L 289 × I 105, were used in these studies. Seeds were soaked for 5 minutes in 1 % sodium hypochlorite solution, thoroughly rinsed with distilled water, and for embryo and shoot material were incubated between wet paper towels in Petri dishes in a dark chamber at 24° C. Seeds used for embryo preparations were incubated for a period of 10 hours, which was found to be sufficient to permit easy removal of the pericarp and excision of the embryo. For the production of etiolated shoots, seeds were incubated for longer periods as indicated in table I. Etiolated shoots were excised at the scutellar node, and thus each shoot consisted of the first internode of the stem, the coleoptile, and the unexpanded foliage leaves and shoot apex within the coleoptile. Since an illuminated growth chamber was not readily available, green seedling material was grown near a window in the laboratory. The seedlings were grown in vermiculite and were watered with tap water. For enzyme preparations, seedlings were excised at the surface of the vermiculite, and the remains of the coleoptiles which clung to the stems were removed and discarded. The term green seedling, as used in this report, therefore refers to only that portion of the seedling which is above the coleoptilar node.

The following procedure was used in making the enzyme preparations. Excised embryos, etiolated shoots, and green seedlings were washed once in cold distilled water and were then thoroughly ground in cold mortars with approximately 5 parts of cold water and 0.7 parts of reagent-grade sea sand. The resulting slurries were diluted with cold water to 10 ml per gm of tissue for the shoot and seedling preparations and 20 ml per gm of tissue for the embryo preparations, and were centrifuged for 2 minutes in a clinical centrifuge. Supernatants from this centrifugation were used as the enzyme preparations. The pH of these supernatants was approximately 7.0 for embryo preparations and 5.8 for preparations of etiolated shoots and green seedlings. The supernatants were used without further dilution for the estimation of catalase, phosphatase, and polyphenolase activities, and were diluted 1 : 5 and 1 : 10 for assay of cytochrome oxidase and peroxidase activities, respectively. For these dilutions pH 7.0 buffer was employed which was 0.05 M with respect to potassium phosphate, 4×10^{-4} M with respect to $AlCl_3$, and 0.41 M with respect to D-mannitol. Preparations were kept in ice baths as much as possible until enzyme runs were completed. Under the conditions used the preparations exhibited generally excellent stability over the period of 4 to 6 hours required for a series of determinations.

In preliminary work on methods of assay for the various enzyme activities, preparations of etiolated shoots were used and conditions, particularly pH and concentrations of substrate and preparation, were varied over wide ranges. The methods which appeared most satisfactory are described below. It was found that when these assay methods were employed, the relationship between reaction rate and preparation concentration approached linearity over a several-fold range in concentration of preparation.

Catalase activity was estimated by a modification of the titrimetric method presented by Sumner and Somers (16). To 7.4 ml of 0.01 M potassium phosphate, pH 7.0, 0.3 ml of 0.21 M H_2O_2 was added, and the solution was allowed to equilibrate in a bath of ice and water (approximately 2° C). Enzyme preparation (0.3 ml) was added at zero time and 2-ml sam-

Table I

Specific Activities of Five Enzymes in Preparations of Embryos, Etiolated Shoots, and Green Seedlings of L 289 × I 205 Corn

Plant part	Age in days	Ave length or ht	Ave fresh wt/part	Protein N/ml original supernatant	Specific activity *				
					Catalase	Cytochrome oxidase	Peroxidase	Phosphatase	Polyphenolase
		mm	*gm*	*mg*					
Embryo	..	...	0.020	0.690	42	0.39	2.0	0.87	0.03
Etiolated shoot	3	4	0.007	0.720	39	0.74	12.2	0.82	0.11
	4	16	0.040	0.380	36	1.09	18.8	1.31	0.31
	5	31	0.080	0.295	38	1.17	27.7	1.66	0.53
	7	76	0.245	0.165	55	1.32	48.8	2.64	0.85
	9	130	0.520	0.105	87	1.35	69.1	3.82	1.43
Green seedling	8	119	0.60	0.220	7	0.44	22.6	1.43	3.21
	11	220	1.06	0.135	15	0.50	36.3	1.91	3.14
	14	280	1.70	0.100	14	0.50	52.6	2.16	4.60

* Specific activities are expressed in the following units per minute per mg protein N:
catalase—micromoles H_2O_2 destroyed
cytochrome oxidase—micromoles cytochrome c oxidized
peroxidase—increase in optical density at 460 mμ
phosphatase—micromoles *p*-nitrophenol liberated
polyphenolase—increase in optical density at 410 mμ

ples were pipetted into 5-ml portions of 2 % H_2SO_4 at ¼, 3¼, and 6¼ minutes. Samples were titrated immediately to the first faint pink with 0.003 M $KMnO_4$ in 2 % H_2SO_4. Although the pink color faded rather rapidly when samples of the reaction mixture were titrated, acceptable reproducibility was attained by titrating to the end point indicated.

Assay of cytochrome oxidase activity was based on the spectrophotometric method of Hogeboom and Schneider (5), the principal change from the published method being a decrease in the concentration of cytochrome c to 1.33×10^{-5} M. Using this lower concentration permitted the use of water in the blank cell of the Beckman DU spectrophotometer, thus making possible measurements at a narrow slit width (0.02 mm). Increasing the concentration of cytochrome c above 1.33×10^{-5} failed to increase the reaction rate under the conditions of these experiments. Cyanide at a concentration of 10^{-3} M effected complete inhibition of activity. In the assay, 2.57 ml of buffer (0.05 M potassium phosphate, 4×10^{-4} M $AlCl_3$, pH 7.0) and 0.4 ml of reduced cytochrome c solution (10^{-4} M), previously brought to 30° C in a constant temperature bath, were mixed in the Beckman cell, 0.03 ml of enzyme preparation was added at zero time, and optical density readings at 550 mμ were recorded at 1-minute intervals for 6 minutes. The cytochrome c used was purchased from the Sigma Chemical Company.

Peroxidase activity was assayed by a method based on the techniques used by Ponting and Joslyn (12) and Morris, Weast, and Lineweaver (11), with the Beckman spectrophotometer cell serving as the reaction vessel. Buffer (0.01 M sodium acetate, pH 5.6) and guaiacol (0.18 M) solutions were brought to 30° C. A 0.5-ml portion of guaiacol was then added to 2.75 ml of buffer in the Beckman cell, 0.2 ml of cold 0.029 M H_2O_2 was added, and at zero time 0.05 ml of enzyme preparation was added. Optical density readings at 460 mμ were recorded at 1-minute intervals over a period of 5 minutes.

Phosphatase activity was assayed by a modification of the method of Sommer (14). To 5.8 ml of buffer (0.05 M citric acid, 0.075 M ethylene diamine, pH 4.9), 1.0 ml of 0.01 M disodium *p*-nitrophenyl phosphate (Sigma Chemical Company) was added, and the solution was brought to 30° C. At zero time 0.2 ml of enzyme preparation was added, and at 1, 5, and 9-minutes, 1-ml aliquots were pipetted from the reaction mixture into 3-ml portions of 0.1 *N* NaOH. Samples were usually allowed to stand approximately 1 hour after addition to the base, and optical densities at 400 mμ were then read using the Beckman DU spectrophotometer. The extinction coefficient of *p*-nitrophenol in dilute base at 400 mμ was found to be 1.60×10^7 cm^2 per mole. This value was used in calculating specific activities. The addition of Mg^{++} to a final concentration of 10^{-3} M increased the phosphatase activity of corn shoot preparations only slightly (< 5 %). It appears, therefore, that the principal corn shoot phosphatase can be included with the class II phosphomonoesterases of Sumner and Somers (16).

Polyphenolase activity was estimated by a method patterned after that of Ponting and Joslyn (12) but adapted for use of the Beckman cell as the reaction vessel. Buffer (0.01 M sodium acetate, pH 5.6) and catechol (0.5 M) solutions were brought to 30° C before they were added to the cell. The reaction mixture consisted of 2.9 ml of buffer, 0.5 ml of catechol, and 0.1 ml of enzyme preparation. The enzyme preparation was added at zero time and optical densities at 410 mμ were recorded at 1-minute intervals for 6 minutes.

In experiments concerned with the centrifugal fractionation of preparations with respect to the five enzyme activities, etiolated shoots were ground in unbuffered 0.41 M mannitol. Following the usual low speed centrifugation, supernatants (pH approximately 5.8) were subjected to centrifugation at approximately $40{,}000 \times g$ for 20 minutes in a Spinco model L refrigerated ultracentrifuge. The sedimented fraction from this centrifugation was washed by resuspending in 0.41 M mannitol and recentrifuging, and was finally suspended in 0.41 M mannitol and diluted to the original volume.

For the determination of protein nitrogen, a modification of the method of Koch and McMeekin (7) was used. Dilutions of the enzyme preparations were treated with equal volumes of 20 % trichloroacetic acid, the resulting precipitates were centrifuged down, washed once with 10 % trichloroacetic acid, and digested with H_2SO_4 and H_2O_2. Nitrogen content of the digests was then determined colorimetrically through the use of Nessler reagent.

Results and Discussion

The results of typical experiments with embryos, etiolated shoots of various ages, and green seedlings of various ages for corn of the single cross hybrid, L 289 × I 205, are presented in table I. Activities listed are based on duplicate determinations. Comparable determinations have been made with embryos and shoots of the variety, Krug, with essentially similar results.

It is apparent from the data that in comparison with shoot preparations, embryo preparations are approximately equal in catalase activity, somewhat lower in cytochrome oxidase and phosphatase activities, and much lower in peroxidase and polyphenolase activities. Seedling preparations, on the other hand, are appreciably lower than shoot preparations in catalase and cytochrome oxidase activities, approximately equal in peroxidase and phosphatase activities, and somewhat higher in polyphenolase activity. In etiolated shoots it appears that there is an increase in the specific activities of the five enzymes with age. Stafford (15) has noted a similar effect of age of etiolated pea seedlings on the activities of several enzymes, one of which was cytochrome oxidase. In the present work, this increase is most pronounced in peroxidase and polyphenolase activities. Some increase in spe-

TABLE II

DISTRIBUTION OF ENZYME ACTIVITIES FOLLOWING HIGH-SPEED CENTRIFUGATION OF A PREPARATION OF 5-DAY ETIOLATED SHOOTS OF KRUG CORN

ACTIVITY	SPECIFIC ACTIVITY *			% TOTAL ACTIVITY			
	CRUDE PREPARATION	SEDIMENT	SUPERNATANT	CRUDE PREPARATION	SEDIMENT	SUPERNATANT	SUM OF SEDIMENT AND SUPERNATANT
Catalase	44.6	84.0	16.1	100	69	21	90
Cytochrome oxidase	1.09	2.48	0.0	100	83	0	83
Peroxidase	32.5	21.1	37.7	100	24	69	93
Phosphatase	1.79	0.40	2.80	100	8	93	101
Polyphenolase	0.73	1.31	0.35	100	66	28	94
Protein N	....	....	....	100	37	59	96

* See footnote, table I, for units.

cific activity with age is noted in green seedling preparations, but the increase found here is generally less than that noted for the shoot preparations. Of particular interest is the fact that embryo preparations are characterized by extremely low peroxidase and polyphenolase activities, while shoots and seedlings—particularly the older ones—yield preparations which are highly active with respect to these two enzymes. As reported by James (6), polyphenolase activity is also very low if not absent in embryos of barley.

The data on protein nitrogen (table I) reveal a decrease in protein content with age in preparations of etiolated shoots and green seedlings. This fact must of course be considered if one is interested in selecting a stage of development which will yield preparations with a high activity per unit volume. The 3-day shoot preparation, for example, is lowest among the shoot preparations in terms of the specific activity of cytochrome oxidase, but it is highest among these preparations in terms of cytochrome oxidase activity per unit volume. Specific activities of the preparations were calculated on the basis of protein nitrogen rather than tissue weight because it is thought that the former means of expression provides a more reliable indication of the enzyme activity per unit of protoplasm.

When crude preparations suspended in 0.41 M mannitol are subjected to centrifugation at 40,000 × g for 20 minutes, a fractionation of activities is effected as indicated in table II. The data presented are based on an experiment with 5-day etiolated shoots of the variety, Krug. Similar results have been obtained with shoots of Krug of other ages, and with shoots of L 289 × I 205. The data show that the sedimented fraction accounts for the bulk of the catalase, cytochrome oxidase, and polyphenolase activities, suggesting that under the experimental conditions used, these enzymes are for the most part associated with cytoplasmic particles. These results agree generally with the work of McClendon (10) who found that in tobacco leaves, cytochrome oxidase activity is to a large extent associated with cytoplasmic particles, and that catalase and polyphenolase activities are at least partially associated with such particles. As shown in table II, the supernatant fraction accounts for most of the phosphatase and peroxidase activities, indicating that if these two activities are associated with any cellular particles, such particles are not readily sedimented under the conditions used. The distribution of catalase and peroxidase activities indicates that in these determinations catalase accounts for little if any of the peroxidase activity, although Tauber (17) has shown that under some conditions catalase can function as a peroxidase.

SUMMARY

Specific activities of preparations of corn embryos, etiolated shoots (excised at the scutellar node), and green seedlings (excised at the coleoptilar node) with respect to five enzymes have been determined. The preparations may be arranged as follows in order of their specific activities: catalase—seedling < shoot, embryo; cytochrome oxidase—embryo, seedling < shoot; peroxidase—embryo < shoot, seedling; phosphatase—embryo < shoot, seedling; and polyphenolase—embryo < shoot < seedling. Etiolated shoots of increasing age yielded preparations of increasing specific activity with respect to the five enzymes. A similar but less pronounced effect of age was noted in experiments with green seedlings. High speed centrifugation of shoot preparations resulted in the sedimentation of a fraction with which were asociated most of the catalase, cytochrome oxidase, and polyphenolase activities of the original preparation, while the supernatant accounted for most of the peroxidase and phosphatase activities.

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THE ISOLATION OF PLANT CUTICLE WITH PECTIC ENZYMES[1,2]

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The isolation of relatively intact sheets of plant cuticle from leaves of several species has been carried out in connection with a study of the interaction of plant cuticle with various foliar applied chemicals. Previous work by Skoss (5) demonstrated that plant cuticle can be isolated by anaerobic bacterial fermentation of leaves. Wood et al (6) observed that filtrates from culture solutions of *Bacterium aroideae* decomposed various plant tissues and sometimes caused the release of cuticle. Chayen (1), Hohl (2), and others have used pectic enzymes as mild macerating agents in anatomical and cytological studies. The present work was an attempt to develop a simplified cuticle isolation procedure based on the use of commercially available pectic enzymes.

Materials and Methods

Disks, 1.8 cm in diameter, were punched with a cork borer from freshly harvested leaves of several species and placed in Griffin beakers. The disks were then covered with approximately 2 ml per disk of buffered pectic enzyme solution containing 100 ppm Merthiolate (Thimerosal, Lilly) as a disinfectant. The disks were weighted to the bottoms of the beakers with heavy wire screen and the enzyme solutions were thoroughly aspirated into the tissues, using intermittent vacuum. The beakers containing the infiltrated disks were then placed on a slow rotary shaker (100 rpm - 2.5 cm radius of rotation) in a cabinet maintained at 35° C. After leaf decomposition, isolated cuticles and vascular tissue were collected by passing the resulting suspension through a 10 mesh wire screen. The isolated cuticle disks and tangled masses of vascular tissue retained by the screen were carefully washed from the screen into a liter of water, gently stirred, and sieved again. Three or four screenings were usually sufficient to remove practically all cellular debris. The leaf vascular tissue was sorted with a tweezers from the washed cuticle disks, and the disks were then stored in distilled water containing 100 ppm Merthiolate. Comparisons between treatments were based on the rates at which isolated cuticles appeared in the decomposition medium.

Experimental Results

Among the several commercial enzyme preparations tested, Enzyme 19 (formerly Enzyme 19AP, Rohm and Haas Co.) and Pectinase (Nutritional Biochemicals Corp.) proved most satisfactory. Pec-

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