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THE EFFECT OF SOME PHENOLIC COMPOUNDS ON THE ACTIVITY OF 6-PHOSPHOGLUCONATE DEHYDROGENASE FROM TOBACCO TISSUE CULTURES

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; phenolic compounds; 6-phosphogluconate dehydrogenase; inhibitors.

Abstract—Anodic polyacrylamide gel electrophoresis of extracts of cultures of tobacco tissue *Nicotiana tabacum* W-38 revealed the presence of two 6-phosphogluconate dehydrogenases (6PGD). The slow and the fast anodic migrating zones were designated I and II, respectively. After purification, enzymes from both zones exhibited no major differences in their affinity towards 6-phosphogluconate (6PG) or $NADP^+$, and were found to have approximately the same pH optima and MWs (69 000–72 000). The coumarins scopoletin and esculetin showed some inhibitory effect on each isozyme at 0.4 mM. Below 0.3 mM, however, esculetin stimulated the activity of zone I when lower amounts of 6PG ($S_{0.25}$) were used. The glucosylated compounds, scopolin and esculin, were much more inhibitory towards the 6PGDs than their respective aglycones. Ferulic, *p*-coumaric and caffeic acids seemed to have an inhibitory effect dependent on 6PG concentration. A larger inhibition was observed in each case at the lower 6PG levels used. Zone I activity appeared to be inhibited to a greater degree than zone II activity by 0.4 mM *p*-coumaric acid with low 6PG. Of the phenolic compounds tested, chlorogenic acid was most effective, completely inhibiting the enzyme activity at 0.4 mM. Of the non-phenolic compounds investigated, glucose 1,6-diphosphate inhibited both isoenzymes of 6PGD at lower 6PG concentrations. On the other hand, 2,3-diphosphoglycerate activated both isoenzymes up to 200% of their original activity.

INTRODUCTION

Scopoletin (6-methoxy-7-hydroxycoumarin) has been reported to be a frequent constituent of solanaceous plants [1]. In the leaves of healthy tobacco plants it occurs mainly as its 7-glucoside, scopolin, along with much smaller amounts of other scopoletin glycosides [2]. Under most stress conditions, the concentration of free scopoletin and/or scopolin increase and both are found in relatively larger amounts than in control plants. Thus, Sequeira [3] found an increase in both scopolin and scopoletin in tobacco plants infected with *Pseudomonas solanacearum* while in boron-deficient tobacco [4], scopolin increased more than 20-fold in the leaves compared to controls. Tobacco plants sprayed with 2,4-dichlorophenoxyacetic acid (2,4-D) also show an increase in both scopolin and scopoletin [5] as do those treated with maleic hydrazide [6]. Other stress conditions known to result in the accumulation of scopolin include certain mineral deficiencies [7], cold treatments [8], UV irradiation [9], and X-ray irradiation [10].

Glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate: $NADP^+$ 1-oxidoreductase, EC 1.1.1.49) (G6PD) is the first enzyme of the pentose phosphate pathway. This enzyme and 6-phosphogluconate dehydrogenase (D-6-phosphogluconate: $NADP^+$ 1-oxidoreductase, EC 1.1.1.44) (6PGD) have been suggested as controlling enzymes of the pathway [11], since the activities of both these dehydrogenases may be limited by the availability of $NADP^+$ and inhibited by NADPH [12]. Erythrose 4-phosphate, an intermediate of the pathway, can combine with phosphoenolpyruvic acid to initiate the shikimic acid pathway, which is used in the biosynthesis of phenylalanine and derived phenolic compounds. Ashihara [13] has suggested a possible role of the pentose phosphate pathway as a supplier of building blocks for phenolic compound biosynthesis and Godin [14] reported that phenolic accumulation results from the enhancement of the pentose phosphate-pathway activity. Farkas [15] has also discussed possible relations between the pentose phosphate pathway activity and the enhanced synthesis of phenolic compounds. In fact, plant infection not only increases phenolic biosynthesis, but also the pentose phosphate pathway [16, 17]. Wender [18] has reviewed which pathways might be enhanced to achieve accumulation of phenolic compounds. The shikimic acid pathway is one such pathway and, furthermore, the formation of phenolics has been reported to be affected by changes in the pool of phenylalanine [19, 20]. The pentose phosphate pathway may have some control over the formation of this aromatic amino acid through the shikimic acid pathway.

Since there appears to be some relationship between the accumulation of scopolin and scopoletin in some plant tissues, and the activity of the pentose phosphate pathway during stress conditions, an investigation of the effect of these and other phenolic compounds on the activity of G6PD isoenzymes from WR-132 tobacco tissue cultures has been reported previously by Hoover *et al.* [21]. This paper reports the results of studies on the effect of various phenolic and non-phenolic compounds on the two

isoenzymes of 6PGD present from W-38 tobacco tissue cultures.

RESULTS

6PGD isolated from tobacco tissue culture W-38 exists as two anodic zones (R_fs: 0.26 and 0.40) on polyacrylamide gel electrophoresis. The slow and the fast migrating zones are designated as zone I and II, respectively.

For kinetic studies, zone I was separated from zone II using a DEAE-cellulose column equilibrated with 0.1 M imidazole buffer, pH 6.5, containing 30 mM mercaptoethanol and 10^{-5} M $NADP^+$ to maintain enzymatic activity. Zone I enzyme assays had to be performed no later than 24 hr after isolation otherwise all activity was lost. Zone II on the other hand was stable for over a week.

For zone I, the plot of pH versus initial velocity yielded an optimal pH value above 7.0 with no change in the activity over the range from 7 to 9. The same was observed with zone II. The $S_{0.5}$ values with respect to either substrate, as determined by a Lineweaver–Burk plot of saturation curve data [22], were also very similar: zones I and II yielded values of 0.087 and 0.078 mM, respectively; for 6PG, and 2.7 and 3.0 μM, respectively, for $NADP^+$. When 6PG was omitted from the assay mixture, no reduction of $NADP^+$ was observed, thus verifying that only the activity of 6PGD was being measured.

The MWs of the proteins of zones I and II were determined by the gel-filtration method of Andrews [23] and the gel electrophoresis method of Weber *et al.* [24]. The gel electrophoresis of enzymes derived from the gel filtration MW method yielded the same results as for zones I and II. The enzymes of zones I and II appear to contain no subunit structure, since both methods yield MWs of 69 000–72 000 for each isoenzyme.

In an attempt to elucidate the possible role of phenolics in regulating the pentose phosphate pathway, several compounds were tested for their effect on the activity of 6PGD from zones I and II, in a manner similar to that of Hoover [21]. The compounds tested were scopolin, scopoletin, esculin, esculetin, as well as the phenolic acids ferulic, caffeic, and *p*-coumaric, and the ester chlorogenic acid. The concentrations of these compounds in the assays were varied from 0.04 to 0.4 mM. The 6PG was also varied from full saturation ($S_{1.0}$) to quarter saturation ($S_{0.25}$) of the enzyme. The reaction solutions were all 5 mM in Mg^{2+} and 0.40 mM in $NADP^+$. The data are shown in Table 1. Although much similarity was found between the 6PGDs of zones I and II in their response to the various phenolic compounds tested, the presence of certain compounds definitely influenced zone I and II activity. For example, scopoletin, at 0.4 mM, was found to have an inhibitory effect on both zones of 6PGD. Esculetin, too, at 0.4 mM, was found to inhibit zones I and II slightly. However, at concentrations below 0.3 mM, it was found that esculetin activated zone I when the lower 6PG concentration ($S_{0.25}$) was used.

The glucosylated compounds, scopolin and esculin, were much more inhibitory towards 6PGD than their aglycones, scopoletin and esculetin. The same pattern of inhibition was found at all the 6PG levels tested. As for the phenolic acids tested, ferulic, *p*-coumaric and caffeic acids seemed to have an inhibitory effect which is dependent on the 6PG concentration. A more potent inhibition was observed in each case at the lower 6PG levels used. Zone I activity appeared to be inhibited to a greater degree (33%) than zone II activity (54%) by 0.4 mM *p*-coumaric acid at the lowest 6PG concentration tested. Of all the compounds tested, chlorogenic acid was the most effective, completely inhibiting the enzyme activity at 0.4 mM.

In an earlier work in our laboratory, Hoover and coworkers [21] found that certain of these phenolic compounds had an inhibitory effect on G6PD from WR-132 tobacco tissue cultures. Our data showed that ferulic, caffeic and *p*-coumaric acids have a more inhibitory effect (up to 50%) on 6PGD from W-38 tobacco tissue culture than on G6PD from WR-132 tobacco tissue culture. Moreover, 6PGD in W-38 was not activated by scopoletin as was G6PD of WR-132 tobacco tissue culture.

Several non-phenolic compounds have also been tested for their effect on 6-phosphogluconate dehydrogenase (Table 1). Glucose 1,6-diphosphate, which others have found to inhibit 6PGD from yeast and several rat tissues [25], inhibited the activity of both zones I and II of 6PGD. The inhibitory effect was more pronounced at the lower 6PG concentrations used. 2,3-Diphosphoglycerate was found to activate the 6PGD up to 200% at the lower substrate concentrations employed. Several other compounds were tested but were found to have no effect on 6PGD. These included 1,6-diphosphofructose, glucose, glucose 1-phosphate, glucose 6-phosphate and borate.

DISCUSSION

6PGD has been found to be inhibited by chlorogenic acid, by certain phenolic acids and by scopolin, scopoletin, esculin and esculetin. Since initial velocities were exclusively used in these studies, the presence of a contaminating enzyme which can convert phenolic compounds to inhibitory compounds is eliminated. With 6PGD, chlorogenic acid was the most inhibitory of the compounds tested. Phenolic acids inhibited the enzyme more effectively when lower 6PG concentrations were used. Scopolin and esculin were more potent inhibitors than their aglucones. At lower concentrations of these phenolic compounds, essentially no inhibitory effect by the latter two was noticed. Thus, the pentose phosphate pathway, the supplier of the building block erythrose 4-phosphate, is a pathway which possibly may be regulated by these phenolic compounds.

The inhibitory effect of glucose 1,6-diphosphate and the stimulatory effect of 2,3-diphosphoglycerate on 6PGD indicate possible influence of these two compounds on the pentose phosphate pathway. Studies, therefore, should be undertaken also to acquire experimental data concerning their possible effect on G6PD activity.

EXPERIMENTAL

W-38 tobacco tissue culture, originally obtained from Dr. Folke Skoog of the University of Wisconsin, was grown on a revised medium of Linsmaier and Skoog [26] using 2 mg/l. indole-3-acetic acid and 200 μg/l. of kinetin. The tissue was grown at room temp. under continual subdued light (1-ft. candle). The inocula were pieces of tissue *ca.* 5 × 5 × 3 mm cut from 3-week-old stock tissue. All operations were performed in a laminar flow hood utilizing sterile techniques.

Disc gel electrophoresis. Enzyme solutions were analyzed for isoenzyme components by polyacrylamide disc gel electrophoresis according to the method of ref. [27]. Gels were composed of 7.5% acrylamide and 0.2% *N,N*-methylene

Table 1. Effect of various phenolic compounds and glucose-1,6-diphosphate and 2,3-diphosphoglycerate on 6PGD zones I and II

Conc. of phenolic compound (mM)	% of control activity											
	$S_{1.0}$6PG		$S_{0.5}$6PG		$S_{0.25}$6PG		$S_{1.0}$6PG		$S_{0.5}$6PG		$S_{0.25}$6PG	
	6PGD I	6PGD II	6PGD I	6PGD II	6PGD I	6PGD II	6PGD I	6PGD II	6PGD I	6PGD II	6PGD I	6PGD II
	Scopoletin						Scopolin					
0.04	100	102	97	95	103	92	93	101	92	98	101	100
0.1	98	102	100	98	103	88	96	100	90	96	97	99
0.2	97	101	94	94	96	86	82	91	78	78	88	80
0.3	88	89	87	95	88	85	33	50	35	50	38	40
0.4	49	67	49	60	51	40	21	21	20	11	13	12
	Esculetin						Esculin					
0.04	104	100	104	85	110	91	102	102	96	102	99	98
0.1	103	100	107	88	112	84	97	101	96	95	101	97
0.2	104	82	102	87	113	81	96	93	88	90	97	88
0.3	102	89	103	84	110	79	60	58	58	55	56	55
0.4	62	78	64	65	79	60	22	20	22	18	17	20
	Ferulic acid						*p*-Coumaric acid					
0.04	99	98	100	95	88	100	100	101	90	90	88	91
0.1	100	89	90	87	81	87	98	100	80	88	83	87
0.2	102	83	84	83	68	72	90	95	74	77	70	72
0.3	90	77	68	72	52	61	89	93	64	65	52	67
0.4	80	67	56	59	45	46	72	80	56	51	33	54
	Caffeic acid						Chlorogenic acid					
0.04	86	95	92	94	96	81	107	103	86	93	90	98
0.1	86	86	92	86	83	75	94	88	82	93	83	88
0.2	82	88	95	77	65	63	81	76	63	68	50	68
0.3	80	83	82	67	54	50	17	26	12	23	11	25
0.4	74	83	79	66	54	49	8	0	0	0	4	0
	Glucose 1,6-diphosphate						2,3-Diphosphoglycerate					
0.04	100	100	100	100	88	95	100	104	121	111	134	150
0.1	100	102	97	95	78	83	105	108	138	129	161	166
0.2	98	100	85	81	60	56	108	110	154	147	181	173
0.3	96	100	64	69	40	43	111	112	165	162	190	192
0.4	89	91	52	59	31	36	118	116	169	178	204	192

bisacrylamide. Runs were made at pH 9.3. Bromophenol blue was added to the upper buffer to serve as a tracking dye. After completion of electrophoresis, the isoenzymes were visualized according to the procedures described in ref. [11] by placing the gels in a soln containing 40 mM Tris–chloride at pH 8.8, 5 mM $MgCl_2$, 250 μM $NADP^+$, 1 mM 6-phosphogluconate (6PG), 0.5 mg/ml *p*-nitroblue tetrazolium, and 25 μg/ml phenazine methosulfate.

MW determinations. MWs of the isoenzymes were determined by gel filtration chromatography using Sephadex G-150 according to the procedure of ref. [23] as modified by Hoover *et al.* [28]. Enzymatic activity was measured to determine the elution vol. of the isoenzymes. MWs of the isoenzymes were calculated using a semi-logarithmic plot of MW vs elution vol.

A second method used for determining MWs was sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis utilizing the procedure of ref. [24] as modified by Hoover *et al.* [28].

After electrophoresis, gels were removed and placed in a staining soln of 0.25% wt/mol, Coomassie Brilliant Blue in 45% methanol and 9% acetic acid for 4–6 hr. The gels were destained with 5% MeOH in 7.5% HOAc until dark protein bands were visible. MWs were calculated from a plot of MWs vs electrophoretic mobility.

Enzyme assays. Only initial velocities were used in these investigations. These initial velocity assays of 6PGD were determined according to the procedure of [29]. All assays were performed with a Varian 635 K UV visible recording spectrophotometer. The assay mixture contained 100 mM Tris–HCl buffer (pH 7.6) and 5 mM Mg^{2+}. Concns of 6PGD and $NADP^+$ varied depending on the assay. Saturating levels of 6PG and $NADP^+$ were 2.62 and 0.40 mM, respectively.

Enzyme preparations. Tissue was mixed with glass beads, washed hydrated polyclar AT and extraction soln (2:1:1:2). The extraction soln consisted of 100 mM Tris–HCl buffer (pH 8.5), 2 mM EDTA and 30 mM mercaptoethanol. The above mixture

was homogenized in a Sorvall Omnimixer at 8000 rpm for 6 min. The Omnimixer cup was immersed in an ice bath during homogenization. The homogenate was filtered through four layers of cheesecloth and centrifuged at 34000 *g* for 15 min. The pellet obtained between the 25% to 70% $(NH_4)_2SO_4$ saturation of the resulting supernatant was dissolved in 20 mM phosphate buffer (pH 7.0) made in 30 mM mercaptoethanol and 10% glycerol. The preparation was dialyzed and then mixed with CM-cellulose (pre-equilibrated with the phosphate buffer) for 30 min. The CM-cellulose was removed by centrifugation. The pellet obtained from a 70% $(NH_4)_2SO_4$ saturation of the resulting supernatant was dissolved in a buffer soln (pH 6.5) containing 30 mM mercaptoethanol and 50 mM imidazole HCl in 10% glycerol. This soln was applied to the top of a DEAE cellulose column which had been pre-equilibrated with the 50 mM imidazole HCl buffer (pH 6.5). Zone I was eluted using this buffer as the eluant. Zone II was then eluted with this buffer made 100 mM in NaCl.

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