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TOTAL PHENOLIC CONTENT AND PEROXIDASE ISOZYMES IN *LINUM USITATISSIMUM*

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Key Word Index—*Linum usitatissimum*; Linaceae; flax; genotypes; peroxidase activity; isozymes; phenolic content; anion exchange resin; statistical analysis.

Abstract—Examination of a method of determining total phenolic content showed that it could be standardized and used on plant extracts from two genotypes of flax. The majority of the phenolic content in these extracts could be removed by adding an anion exchange resin during extraction and dialysing the resulting extracts. Examination of phenolic content and the activities and relative mobilities of peroxidase isozymes in both genotypes revealed effects of phenolic removal on these isozyme characteristics; such effects differed between the genotypes.

INTRODUCTION

ONE OF the most important functions of phenolic compounds in plant tissues may be the metabolic regulation of red-ox potentials.[1,2] Preferential oxidation of phenolics by peroxidase may prevent the peroxidase-catalysed oxidation of indole-acetic acid (IAA). A similar mechanism may also regulate the other enzymes of the IAA-oxidase system.[3] Phenolic compounds are abundant in plant tissues and their oxidation must itself be regulated. It has been suggested that excess phenolics are protected from oxidation by the soluble enzyme fraction either by being spatially separated from it, possibly in the vacuoles,[4] or stored in inactive form as glycoside derivatives.[5] However, this protection is disrupted during extraction of the soluble enzyme fraction and the phenolic compounds interfere with many cell free reactions. On disruption of the cell organization phenolics may be oxidized to quinones, condensed tannins or brown pigments which also inactivate enzymes and may cause precipitation of soluble proteins.[4] In addition phenolic compounds in plant tissue extracts react with Folin–Ciocalteu reagent causing errors in total protein content estimation by the Lowry method.[6] A method for determining protein content in the presence of phenolics has been reported.[7] However, only the removal of the phenolics, or inhibition of their oxidation by the addition of reducing agents, enables the accurate estimation of the activity of particular enzymes.

The high phenolic content in flax interferes with studies of peroxidase activity and the determination of protein content. Although the phenolic content and its relationship to peroxidase activity is important, removal of phenolics from the soluble enzyme fraction

[1] STONIER, T., HUDECK, J., VANDE-STOUWE, R. and YANG, H. (1970) *Physiol. Plant.* **23**, 775.
[2] STONIER, T., SINGER, R. W., and YANG H. (1970) *Plant Physiol.* **46**, 454.
[3] PSENAK, M., JINDRA, A. and KOVACS, P. (1970) *Biol. Plant.* **12**, 241.
[4] ANDERSON, J. W. (1968) *Phytochemistry* **7**, 1973.
[5] HARBORNE, J. B. (1964) in *Biochemistry of Phenolic Compounds* (HARBORNE, J. B., ed.), p. 129, Academic Press, London.
[6] LOWRY, O. H., ROSENBROUGH, N. J., FARR, A. L. and RANDALL, R. J. (1951) *J. Biol. Chem.* **193**, 265.
[7] POTTY, V. H. (1969) *Anal. Biochem.* **29**, 535.

is necessary. The use of reducing agents is not advantageous. Lam and Shaw[8] have shown that a Dowex anion exchange resin is more effective in removing phenolics than the commonly used insoluble polyvinylpyrolidone. The relative efficiency of a Dowex resin and the dialysis technique for the removal of phenolics is reported here. The removal of phenolics can be examined as changes in peroxidase activity and total protein content or, more preferably, by the quantitative determination of total phenolic content. The method of Nair and Vining,[9] used by Lam and Shaw,[8] for determining phenolic content has been examined and a standardized technique is given here. In addition the relationship between the phenolic content, the peroxidase activity and the peroxidase isozymes of two genotypes of flax are considered.

RESULTS

The method for determining phenolic content, using Pauly's reagent,[9] depends on the reaction of the free OH groups of phenolic compounds with diazotized sulphanilic acid ($C_6H_4N_2O_3S$) to form a coloured azo compound. The $C_6H_4N_2O_3S$ is initially reacted with an acidic phenolic solution to produce an intermediate product and the final reaction product is formed when the reaction mixture is made alkaline with Na_2CO_3 solution. It was found that, although the reagents used to synthesize the $C_6H_4N_2O_3S$ were stable at 4° for 2–3 days, the $C_6H_4N_2O_3S$ was unstable; with slow preparation it could be used for up to 3 hr if kept at 4°.

The stabilities of the intermediate and final reaction products were examined using standard solutions of chlorogenic acid at 2 concentrations (1000 and 2000 μg/ml). Spectrophotometric scans showed that the absorbance peak on the intermediate product had a λ_{max} between 400 and 412 nm and that its absorbance stabilized over the first 10–15 min after addition of the $C_6H_4N_2O_3S$. The λ_{max} of the final product reached stability after 6 min when the Na_2CO_3 solution was added 10–15 min after the $C_6H_4N_2O_3S$, and was between 410 and 425 nm. Addition of the Na_2CO_3 solution 30 min after the $C_6H_4N_2O_3S$ resulted in an asymmetrical absorbance curve with λ_{max} at 410–425 nm and a second smaller peak at approximately 479 nm. The final product remained stable for up to 3 hr.

Addition of the Na_2CO_3 solution caused effervescence in the reaction mixture but wetting agent (Tween 80) in the solution prevented bubbles from adhering to cuvette walls.

Standard solutions of cinnamic acid and the cinnamic acid derivatives, chlorogenic, caffeic and ferulic acids were assayed. As cinnamic acid lacks an OH group, no reaction occurred. The monophenolic derivative, ferulic acid, produced a reaction product with λ_{max} at approximately 470 nm while the λ_{max} of the diphenolics, chlorogenic and caffeic acids, were at approximately 420 and 435 nm respectively. Assays of extracts from the two flax genotypes, *R* and *M*, indicated that the λ_{max} in these plant extracts was at approximately 420 nm. The precise position of all λ_{max} was found to depend on the phenolic concentration being examined. λ_{max} decreased slightly with increasing concentration of the diphenolics and increased slightly with increasing concentration of the monophenolic and the plant extracts. The absorbance curves from assays of plant extracts were found to be less symmetrical than those of pure phenolics; at high wavelengths the sides of the curves were flatter.

Within the absorbance range 0·00–2·00 the relationship between concentration of phenolic and reaction product was linear for all 3 phenolics examined. Chlorogenic acid

[8] LAM, T. H. and SHAW, M. (1970) *Biochem. Biophys. Res. Commun.* **39**, 965.
[9] NAIR, P. M. and VINING, L. C. (1965) *Phytochemistry* **4**, 161.

was chosen as a standard and 420 nm used as constant wavelength for estimating phenolic content for chlorogenic acid and plant extracts. The mean absorbance values at 420 nm of duplicate assays on 6 concentrations of chlorogenic acid, between 0 and 2000 μg/ml, were plotted against concentration. The linear regression fitted to the standard curve was highly significant with a slope $b = 1{\cdot}5189 \pm 0{\cdot}0401$. Similar assays were carried out on plant extracts, from both genotypes, at 5 dilutions. The linear regressions of A_{420} on extract concentration were significant for both genotypes and t-tests showed that neither of the slopes were significantly different from that of the standard curve. At high concentrations of plant extract, resulting in absorbance values greater than 0·85, it was observed that the slopes for both genotypes started to flatten off. Linear regressions on the 4 lower concentrations of plant extracts from R and M gave slopes of $b_R = 1{\cdot}5288 \pm 0{\cdot}2313$ and $b_M = 1{\cdot}4700 \pm 0{\cdot}2255$, indicating that within the absorbance range 0·00–0·85 the chlorogenic acid standard curve was reliable for estimating the phenolic content present in plant extracts; this absorbance range corresponded to 0–1150 μg/ml chlorogenic acid.

A standard solution of protein, BSA, was assayed. The reaction of the free OH groups in protein was negligible; a sample of 0·25 mg/ml protein produced a reaction product equivalent to 130 μg/ml of chlorogenic acid.

TABLE 1. PEROXIDASE ACTIVITY (ΔA/min/unit fr. wt) IN DIALYSED AND UNDIALYSED PLANT EXTRACTS FOR TREATMENTS WITH DOWEX ANION EXCHANGE RESIN

		Resin treatment			
Genotype	Sample	(a) 15% at homogenization	(b) 15% after homogenization	(c) 30% at homogenization	(d) No resin
R	Undialysed	0·198	0·182	0·157	0·081
	Dialysed	0·298	0·255	0·257	0·184
M	Undialysed	0·146	0·147	0·141	0·092
	Dialysed	0·288	0·219	0·233	0·166
Totals		0·903	0·803	0·788	0·523

A sample of 16-day-old material, from both R and M was homogenized in buffer, pH 7·0, and in each of the following conditions; (a) 15% resin was added before homogenization, (b) 15% resin was added after homogenization, (c) 30% resin was added before homogenization, and (d) no resin was added, and a dialysed sample of each was prepared. The peroxidase activity of the dialysed and undialysed samples was assayed and the results are shown in Table 1. Dialysis and the addition of resin both resulted in highly significant increases in peroxidase activity. Duncan's multiple range test on the 4 totals from conditions (a)–(d) showed that comparison of condition (b) with (c) was not significant but that all other comparisons were highly significant.

Sixteen-day-old stem tissue of R was ground in buffer, pH 7·0, with 0, 10, 20, 30, 40 and 50% (w/v buffer) resin added before homogenization. The resulting extracts were divided in half, the resin was removed from the first half after 1·5 hr and from the other half on the following day. The results of peroxidase activity assays on the undialysed extracts are given in Table 2. Analysis of these data showed that there was no significant effect of the

time at which the resin was removed, that there was a highly significant increase in peroxidase activity when up to 20% resin was used and that above 20% the amount of resin resulted in no further increase in peroxidase activity. This experiment was repeated using a larger mesh resin, Dowex anion exchange resin 1-X8 50–100 mesh. Analysis of the data showed a highly significant effect of time; the mean peroxidase activity when the resin was removed after 1·5 hr was 73% of that if removed the following day. In addition, as with the smaller mesh, the peroxidase activity was highest in the 20 and 30% extract. However, at concentrations higher than 30% there was a significant decrease in peroxidase activity.

TABLE 2. PEROXIDASE ACTIVITY (ΔA/min/unit fr. wt) FOR GENOTYPE *R* HOMOGENIZED WITH DOWEX ANION EXCHANGE RESIN

Resin %	Resin removed after 1·5 hr	Resin removed following day	Totals	Resin %	Resin removed after 1·5 hr	Resin removed following day	Totals
0	0·089	0·089	0·178	30	0·205	0·213	0·418
10	0·177	0·196	0·373	40	0·198	0·214	0·412
20	0·194	0·219	0·413	50	0·216	0·196	0·412

Tissue from 30-day-old plants of R and M was homogenized in the presence of 20% resin in 0·1 M buffer at pH values of 5·0, 7·0 and 8·0, and a sample from each was dialysed against its corresponding homogenizing buffer. The peroxidase activities of the dialysed and undialysed samples are given in Table 3. The analysis of variance for these data revealed highly significant effects of dialysis and pH on peroxidase activity, which was highest for both genotypes in the dialysed samples at pH 8·0. The corresponding values for phenolic content in these samples are also shown in Table 3. Their analysis showed a highly significant decrease in phenolic content as a result of dialysis and, while the overall effect of

TABLE 3. COMPARISON OF THE EFFECTS OF HOMOGENIZING BUFFER pH ON PEROXIDASE ACTIVITY (ΔA/min/unit fr. wt) AND PHENOLIC CONTENT (μg/ml CHLOROGENIC ACID)

Genotype	pH	Phenolic content	Peroxidase activity	Genotype	pH	Phenolic content	Peroxidase activity
R	5·0	90	0·103	*M*	5·0	420	0·160
Undialysed	7·0	200	0·145	Undialysed	7·0	550	0·220
	8·0	385	0·202		8·0	550	0·233
	5·0	35	0·196*		5·0	110	0·255
Dialysed	7·0	55	0·238	Dialysed	7·0	135	0·333
	8·0	105	0·308		8·0	150	0·363

* Data estimated by missing plot procedure.

pH was not significant, the break-down of the pH sums of squares showed that there was a significant increase in phenolic content between pH 5·0 and 8·0. Table 4 gives the mean protein content of extracts of *R* and *M* at pH values of 7·0 and 8·0, and shows that there was a significant decrease in the protein content, or amount of reaction product produced by the Lowry reaction, between pH 7·0 and 8·0.

TABLE 4. PROTEIN IN DETERMINATIONS (A_{750}) ON TCA PRECIPITATIONS OF EXTRACTS FROM GENOTYPES *R* AND *M* HOMOGENIZED IN BUFFER AT 2 pHs

Genotype	pH	TCA precipitation	Mean absorbance (2 assays)	Mean absorbance (over assays and precipitations)	Corresponding protein concn mg/ml	*t* for differences between the pH means
R	7·0	*A**	0·903 ± 0·023	0·893 ± 0·013	0·429	$t_6 = 3{\cdot}932$†
		B	0·883 ± 0·018			
	8·0	*A*	0·773 ± 0·058	0·776 ± 0·027	0·373	
		B	0·780 ± 0·030			
M	7·0	*A*	0·555 ± 0·006	0·544 ± 0·010	0·261	$t_6 = 8{\cdot}937$†
		B	0·532 ± 0·018			
	8·0	*A*	0·228 ± 0·001	0·277 ± 0·028	0·133	
		B	0·325 ± 0·005			

* Where *A* and *B* represent separate TCA precipitates.
† Significant at probability <0·01.

The effects of homogenizing buffer pH on the activities and relative mobilities (R_ms) of the peroxidase isozymes, in dialysed and undialysed samples of both genotypes, were examined. The isozyme patterns were similar in all samples and the activities and R_ms of 3 slow-migrating, low activity bands and a fourth high-activity, fast-migrating band were measured. The mean isozyme activities and R_ms of the genotypes at each pH are given in Table 5, and the analyses of variance for activity and R_m are summarized in Tables 6 and 7 respectively.

TABLE 5. MEAN PEROXIDASE ACTIVITY (PEAK AREA, in²) AND MEAN R_m OF PEROXIDASE ISOZYMES FOR GENOTYPES *R* AND *M* HOMOGENIZED IN BUFFER AT 3 pHs

Genotype		pH	Isozymes 1	2	3	4	Totals
Mean activity (in²)	*R*	5·0	0·60	1·37	0·52	4·94	7·43
		7·0	0·61	1·22	0·41	9·13	11·37
		8·0	1·94	3·41	1·42	14·59*	21·36
		Means	1·05	2·00	0·78	9·55	
	M	5·0	0·89	0·36	0·67	16·09	18·01
		7·0	1·21	1·00	1·06	16·95	20·22
		8·0	0·78	0·53	0·90	17·48	19·69
		Means	0·96	0·63	0·88	16·84	
Mean relative mobility	*R*	5·0	0·085	0·156	0·204	0·441	0·886
		7·0	0·082	0·154	0·196	0·443	0·875
		8·0	0·082	0·155	0·193	0·438	0·868
		Means	0·083	0·155	0·198	0·441	
	M	5·0	0·087	0·188	0·223	0·455	0·953
		7·0	0·085	0·186	0·217	0·454	0·942
		8·0	0·094	0·184	0·222	0·450	0·950
		Means	0·088	0·186	0·221	0·453	

* The isozyme activities in the dialysed sample used in this mean were estimated by missing plot procedure.

For both activity and R_m the highly significant main effects of genotypes and isozymes and their first order interaction demonstrate the differences between these genotypes which have been reported previously.[10] For activity, the first order interactions between genotypes

[10] FIELDES, M. A. and TYSON, H. (1972) *Can. J. Genet. Cytol.* **14**, 625.

TABLE 6. ANALYSIS OF VARIANCE OF ISOZYME ACTIVITY (in^2) FOR GENOTYPES *R* AND *M* HOMOGENIZED IN BUFFERS AT 3 pHs, UNDIALYSED AND DIALYSED

	df	MS	F
Total	47	34·1333	
Genotypes (Geno)	1	26·6844	73·462†
Isozymes (Iso)	3	441·2703	1214·784†
Dialysis (Dial)	1	0·1326	NS
pH	2	14·7915	40·720†
First order interactions			
Geno × Iso	3	46·8229	128·900†
Geno × Dial	1	3·4524	9·504*
Geno × pH	2	10·3246	28·423†
Iso × pH	6	5·3801	14·807†
Iso × Dial	3	1·5902	NS
Dial × pH	2	0·5679	NS
Second order interactions			
Geno × Iso × pH	6	2·8948	7·969*
Remainder	11	0·1589	NS
Error (Third order interaction)	6	0·3633	

NS Not significant at probability 0·05.
* Significant at probability 0·05–0·01.
† Significant at probability < 0·01.

and pH and between isozymes and pH, together with the significant second order interaction, reflected different responses of the isozymes to pH both within and between genotypes. In both genotypes the activity of isozyme 4 increased with pH while in isozymes

TABLE 7. ANALYSIS OF VARIANCE OF ISOZYME RELATIVE MOBILITY FOR GENOTYPES *R* AND *M* HOMOGENIZED IN BUFFERS AT 3 pHs, UNDIALYSED AND DIALYSED

	df	MS	F
Total	47	0·018518	
Genotypes (Geno)	1	0·004094	346·602†
Isozymes (Iso)	3	0·287774	24362·804†
Dialysis (Dial)	1	0·000425	36·016†
pH { 5·0–8·0	1	0·000089	7·516*
pH { 7·0–(5·0 + 8·0)	1	0·000025	NS
First order interactions			
Geno × Iso	3	0·000401	33·971†
Dial × pH	2	0·000070	5·935*
Remainder	12	0·000025	NS
Second order interactions			
Geno × Iso × Dial	3	0·000070	5·935*
Iso × Dial × pH	6	0·000052	5·362*
Remainder	8	0·000020	NS
Error (Third order interaction)	6	0·000012	

NS Not significant at probability 0·05.
* Significant at probability 0·05–0·01.
† Significant at probability < 0·01.

1, 2 and 3 the activity was highest at pH 8·0 for *R* and at pH 7·0 for *M*. The sums of the isozyme activities, total activities, in the undialysed and dialysed samples are given in Table 8. Dialysis increased the total activity in *R* and had little effect on *M*. Over both genotypes there was a significant correlation ($r_{10} = 0{\cdot}6266$) between total activity, after electrophoretic separation, and gross activity, before separation. The correlations of the corresponding gross activities with the activities of individual isozymes on the genotypes separately showed however, that only those of isozymes 1, 2 and 3 in *R* were significant. The R_m analysis showed a significant decrease in R_m from pH 5·0 to 8·0 and from undialysed to dialysed samples. The total R_ms of the four isozymes, given in Table 8, and the interaction between dialysis and pH indicated that, with increasing pH, R_m decreased in undialysed and increased in dialysed samples. Further examination of the data revealed that this held for isozyme 4 in undialysed samples of *M* and in dialysed samples of *R*, and that the R_m totals for these samples shown in Table 8 reflected the behaviour of isozymes 1, 2 and 3. These isozymes differences gave rise to the significant second order interactions.

TABLE 8. TOTAL ACTIVITY AND R_m OF 4 ISOZYMES OF GENOTYPES *R* AND *M* HOMOGENIZED IN BUFFER AT 3 pHs, UNDIALYSED AND DIALYSED

		R		*M*	
	pH	Total activity	R_m sum over 4 isozymes	Total activity	R_m sum over 4 isozymes
Undialysed	5·0	6·15	0·901	19·22	0·976
	7·0	10·82	0·897	21·79	0·946
	8·0	19·19	0·865	19·48	0·954
Dialysed	5·0	8·72*	0·872	16·79	0·930
	7·0	12·21	0·849	18·66	0·937
	8·0	22·93	0·862	19·88	0·946

* Total of isozyme activities estimated by missing plot procedure.

The relative effects of dialysis and incorporation of resin, prior to homogenization, on peroxidase activity and phenolic content were examined. Plant extracts of both genotypes in buffer pH 8·0 were prepared with and without resin and a sample of each was dialysed. The peroxidase activities and phenolic contents are given in Table 9. Dialysis and resin were both found to significantly increase peroxidase activity while significantly decreasing the phenolic content. The correlation between peroxidase activity and phenolic content was negative but not significant ($r_6 = -0{\cdot}6622$), and for phenolic content there was a highly significant interaction between dialysis and resin. The data revealed that the effects of dialysis and resin treatment were additive for increasing peroxidase activity, and that the decrease in phenolic content as a result of dialysis was not as great in samples which had been treated with resin as in those treated by dialysis alone. However, the phenolic contents of samples after resin treatment and dialysis indicated that the majority of the phenolics had been removed, and the remaining reaction product could be attributed to the protein content of the extracts.

Electrophoretic scans of these samples showed that resin treatment and dialysis did not alter the basic peroxidase isozyme patterns. The total activities of isozymes 1, 2 and 3 and the activities of isozyme 4 are shown in Table 10. The analysis of variance of these data, using the pooled second and third order interactions to obtain the error mean square,

TABLE 9. COMPARISON OF THE EFFECTS OF TREATMENT WITH DOWEX ANION EXCHANGE RESIN AND DIALYSIS ON PEROXIDASE ACTIVITY (ΔA/min/unit fr. wt) AND PHENOLIC CONTENT (μg/ml CHLOROGENIC ACID)

Genotype	Treated (+) or untreated (−) with resin	Phenolic content	Peroxidase activity
R			
Undialysed	−	1060	0·186
	+	385	0·202
Dialysed	−	255	0·212
	+	105	0·308
M			
Undialysed	−	1105	0·164
	+	550	0·233
Dialysed	−	325	0·180
	+	150	0·363

showed the expected highly significant differences between genotypes and isozymes and their interaction. Furthermore, there was a highly significant increase in activity as a result of dialysis which was more marked in *R* than *M* and resulted in a significant genotype by dialysis interaction. The difference between samples treated and untreated with resin was not significant; however, a significant interaction between isozymes and resin treatment revealed that, in both genotypes, resin treatment increased the total activity of isozymes 1, 2 and 3 and had little effect on isozyme 4. Further, the correlation coefficient for total isozyme activity of isozymes 1, 2 and 3 with gross peroxidase activity was significant ($r_6 = 0{\cdot}8052$) while that of isozyme 4 with gross activity was not significant. Analysis of the R_ms of isozymes 1, 3 and 4 (the R_m of isozyme 2 could not be reliably measured in the lower activity samples of *M*) indicated that dialysis and resin treatment resulted in R_m shifts in all 3 isozymes, as can be seen in Table 11. There was a tendency for both dialysis and resin treatment to increase R_m in *R* and to decrease R_m in *M*. In particular, dialysis significantly increased the R_m of isozymes 3 and 4 of *R*, and resin treatment significantly decreased the R_m of isozyme 4 in genotype *M*. Examination of the undialysed and dialysed

TABLE 10. COMPARISON OF THE EFFECTS OF TREATMENT WITH DOWEX ANION EXCHANGE RESIN AND DIALYSIS ON THE PEROXIDASE ACTIVITY (in^2) OF ISOZYMES 1, 2 AND 3 AND ISOZYME 4

Genotypes	Treated (+) or untreated (−) with resin	Total activity isozymes 1, 2 and 3	Activity isozyme 4	Totals
R				
Undialysed	−	2·025	14·470	16·495
	+	4·425	11·945	16·370
Dialysed	−	7·458	18·660	26·118
	+	8·720	17·525	26·245
M				
Undialysed	−	3·435	19·350	22·785
	+	4·380	20·265	24·645
Dialysed	−	4·795	21·650	26·445
	+	8·440	21·675	30·115

data at pH 8·0, in the other experiment showed that, with the exception of isozyme 3, dialysis increased the R_ms in *R*; with the exception of isozyme 4, dialysis decreased the R_ms in *M*.

Plant extracts which had not been homogenized with resin or dialysed were discoloured by oxidized phenolics. This discolouration precipitated with the proteins during TCA precipitation. Protein determinations on these precipitates were found to be inconsistent within and between precipitates from the same sample. Further the presence of the oxidized phenolics resulted in falsely elevated protein estimates. When the extracts were prepared using resin and dialysis the repeatibility of protein estimations was improved. Table 4 gives the mean values of two protein assays on two TCA precipitates, *A* and *B*, for both genotypes at pHs 7·0 and 8·0. The standard errors of the means indicate the reproducibility of these assays within and between precipitates. With one exception (*R*, *A*, pH 8·0, $S = 0{\cdot}058$), these standard errors were of the same order as those obtained for the means of duplicate assays on standard BSA.

TABLE 11. COMPARISON OF THE EFFECTS OF TREATMENT WITH DOWEX ANION EXCHANGE RESIN AND DIALYSIS ON RELATIVE MOBILITY OF PEROXIDASE ISOZYMES 1, 3 AND 4

Genotype	Isozyme 1	3	4	Totals
R				
Undialysed	0·085	0·215	0·495	0·795
Dialysed	0·093	0·240	0·528	0·861
− Resin	0·086	0·224	0·521	0·831
+ Resin	0·093	0·240	0·528	0·861
M				
Undialysed	0·090	0·243	0·512	0·845
Dialysed	0·091	0·239	0·500	0·830
− Resin	0·090	0·243	0·512	0·845
+ Resin	0·091	0·233	0·484	0·808

DISCUSSION

Ibrahim and Shaw[11] have reported that the major phenolic constituents in the cotyledons and young shoots of flax consist of esters and glycosides of caffeic acid derivatives, especially chlorogenic acid, and flavone-C-glycosides. Using Pauly's reagent[9] to measure the total phenolic content in flax, a linear response of absorbance on concentration for a number of cinnamic acid derivatives, including chlorogenic acid, and with plant extracts, was obtained. The absorbance curves obtained when assaying plant extracts of this species resembled those obtained with chlorogenic acid, with λ_{max} at 420 nm, but lacked the symmetry of the chlorogenic acid curve. This suggested the presence, in the plant, assays of reaction products of other phenolics, possibly the monophenolic derivatives of caffeic acid.

Loomis and Battalie[12] reviewed the interactions which may occur between phenolic compounds and proteins, with reference to the inhibition of enzyme activity. Phenolic compounds can bind to protein by hydrogen bonding or may be oxidized to quinones which copolymerize with proteins through covalent bonding. Although the complex role of peroxidase and phenolics in the regulation of IAA is not fully understood[2,13] this regula-

[11] IBRAHIM, R. K. and SHAW, M. (1970) *Phytochemistry* **9**, 1855.
[12] LOOMIS, W. D. and BATTAILE, J. (1966) *Phytochemistry* **5**, 423.
[13] KENTEN, R. H. (1955) *Can. J. Botany* **59**, 110.

tion may be due in part to the inhibition of peroxidase activity by phenolic compounds.[3,14] Comparison of the peroxidase activity and the phenolic content showed here, in flax, that both the incorporation of Dowex anion exchange resin, during the preparation of plant extracts, and dialysis of the extracts reduced the phenolic content and increased the peroxidase activity. Optimal removal of phenolics was achieved with 20% (dry wt/vol. buffer) resin followed by dialysis. The absorption of phenolics was found to be faster with 200–400 mesh resin than with the larger 50–100 mesh resin which would minimize the possibility of the phenolics oxidizing or forming stable phenolic-protein complexes.

Comparison of homogenizing buffers at pHs 5·0, 7·0 and 8·0 showed that the gross peroxidase activity, before electrophoresis, and the phenolic content were higher at pH 8·0. At this pH the phenolics and the proteins would be more negatively charged, which would prevent hydrogen bonding between them and permit them to react better with their corresponding substrates. This prevention could also account for the lower protein content estimates on samples at pH 8·0 compared to pH 7·0 after TCA precipitation. With less association between phenolics and proteins a greater proportion of the remaining phenolics would be expected to be removed by dialysis at pH 8·0 than at the other pHs. However, at pH 8·0, being more ionized, the phenolic compounds might be expected to oxidize more readily to quinones which could bind in a more stable form to the proteins. It was found that in genotype *R* the proportion of phenolics removed by dialysis was greater at pH 8·0 than at the other pHs, whereas in genotype *M* the proportion removed was slightly greater at pH 7·0. The gross peroxidase activity and the activities of all four isozymes of *R* were highest at pH 8·0, and, although the gross peroxidase activity of *M* was highest at pH 8·0, the activities of the isozymes of *M* showed that this reflected the activity of isozyme 4 and that the activities of the other isozymes were highest at pH 7·0. Furthermore, at pH 8·0 the effect of dialysis on the isozyme activities was much greater in genotype *R* than in genotype *M*. It is possible therefore that at pH 8·0 the phenolic compounds were less strongly bound to the peroxidase isozymes of *R* than to those of *M*, particularly isozymes 1, 2 and 3. This would suggest that in genotype *M* the phenolics were more oxidized at pH 8·0 than at pH 7·0.

Brown and Wright[15] have shown that interactions between the phenolic compounds in tea infusions and milk proteins altered the electrophoretic mobilities of the milk proteins, and that the interactions may be the result of hydrogen bonding between the phenolics and the proteins. The changes in MW and in net charge resulting from the interaction of phenolic compounds and proteins which cause interactions of the R_ms of the proteins could be very complex. Hydrogen bonding of phenolics to protein and copolymerization of oxidized phenolics with protein might be expected to decrease the R_m because of the increase in MW. However, with hydrogen bonding the change in net charge of the protein would be very small, but, on the assumption that positively polarized hydrogens on the phenolics bond with negatively polarized hydrogens of the protein, the net charge of protein would be slightly less negative in the presence of phenolics, while with covalent bonding between oxidized phenolics and protein there would be a decrease in the net positive charge of protein in the presence of phenolics. Free protein would be expected, therefore, to have a higher R_m than a phenolic-protein complex formed by hydrogen bonding, and a lower R_m than a covalently bound phenolic-protein complex. With substituted phenolics there remains the possibility that the side group itself carries a charge which could alter the R_m of

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the phenolic-protein complex. The observation here that the removal of phenolics, by resin treatment and dialysis, increased the R_m of isozymes in genotype *R* and decreased the R_m of isozymes of genotype *M* would be in line with the suggestion that at pH 8·0 the phenolic compounds are more covalently bound to the proteins in *M* than in *R*. The behaviour of the R_ms, of the isozymes in both genotypes, in buffers of different pHs would appear to be more complex. In the absence of phenolics the R_ms would be expected to increase with increasing pH, as is the case in dialysed samples.

The results presented here indicated that the major component of the phenolic content of flax could be removed from plant extracts and that the relationship between the phenolic content and the peroxidase enzyme in two flax genotypes, whilst complex, might account for observed alterations in the activities and relative mobilities of the peroxidase isozymes. As suggested elsewhere,[14] this relationship would appear to be different in the two genotypes, and also to some extent, between the isozymes within the genotypes.

EXPERIMENTAL

Plant culture. Two varieties of *Linum usitatissimum*, linseed genotype Royal (*R*) and fibre genotype Mandarin (*M*), were grown for 16 or 30 days in vermiculite to which constant volumes of inorganic nutrients[16] were added at regular intervals. The seedlings were maintained in a constant environment chamber with a 20 hr day length and a 24 hr temperature cycle ranging from 14–22°. The light intensity at the plant apices was *ca.* 54 000 lx, supplied from fluorescent and tungsten lamps.

Extraction and analysis. Stem tissue was homogenized in 0·1 M $Na_2HPO_4.7H_2O$–KH_2PO_4 buffer (pH 5·0, 7·0 or 8·0) in the ratio 1 g tissue (fr. wt) to 4 ml buffer. Where used, Dowex anion exchange resin, 1-X8, 200–400 mesh was equilibrated in phosphate buffer, dried and a percentage (dry wt/vol. buffer) added prior to homogenization. Extracts were strained through nylon mesh, maintained at room temp. 2–3 hr, frozen, rethawed and centrifuged 20 min at 45 000 *g*, and the supernatants recentrifuged 15 min at 7000 *g*. Dialysed samples were prepared by dialysing 1 ml samples of plant extracts against 80 ml of homogenizing buffer for 18 hr. All extracts were stored at −17°. Gross peroxidase activity (activity prior to electrophoresis) was estimated by the method of Maehly and Chance[17] as described previously.[18] The plant extracts were diluted ten-fold with buffer pH 7·0 before assay and the gross peroxidase activities (mean values from 2 assays) recorded as ΔA_{470}/min/unit fr. wt. Protein content was determined by the Lowry[6] method after precipitation in 6·6% trichloro-acetic acid (TCA). The precipitates were washed in TCA, EtOH, Et_2O and acetone, redissolved in 1 N NaOH and dil. 0·25 in 1·0 with dist. H_2O. Optimum results for the determination of total phenolic content were attained using the following procedure: to prepare Pauly's reagent, 20 mg of sulphanilic acid in 10 ml of 0·1 N HCl was cooled and cold solution of 8 mg of $NaNO_2$ in 1·6 ml of H_2O was added dropwise, with stirring, keeping the temperature below 8°. The plant extracts to be assayed were diluted 0·2 in 1·0 with 0·1 M $C_6H_8O_7H_2O$–0·2 M $Na_2HPO_4.7H_2O$ buffer at pH 4·6. The standard solutions of the phenolic compounds were prepared in buffer at pH 4·6. Equal volumes of the solution to be assayed, Pauly's reagent and a 20% Na_2CO_3 solution, containing 1 drop/100 ml of Tween 80 were reacted. The Na_2CO_3 solution was added precisely 10 min after the Pauly's reagent. After a further 20 min the concentration of reaction product was measured by scanning between 320 and 700 nm against a blank reaction mixture. The phenolic content was expressed as absorbance at λ_{max} and for plant extracts the phenolic content (μg/ml) was estimated using a chlorogenic acid standard curve.

Electrophoresis. A detailed description of the method employed for the electrophoretic separation of the anodally migrating peroxidase isozymes has been given elsewhere.[18] Plant extracts, diluted 0·66 in 1·0 by a solution of sucrose and bromophenol blue, were applied to the gel tracks in 0·05 ml aliquots. The electrophoresis cell was run at 400 V until the boundary markers had travelled 15 cm. The isozymes were stained by supplying a H_2O_2 substrate with guaiacol and buffer 7·0.[18] The gel tracks were scanned at 295 nm and the absorbance output recorded. The peroxidase activity of each isozyme was expressed as the area under its absorbance peak. Mean relative mobilities (R_ms) and isozyme activities were calculated from duplicate gel tracks.

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