Theoretical and Applied Genetics 41, 136–141 (1971)

Peroxidase Isoenzymes in *Linum*

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Summary. Isoenzymes of peroxidase were separated on acrylamide gels in 2 genotypes of *Linum usitatissimum* L. and their F_1, F_2 and first backcross progeny. Active extracts were obtained from homogenates of main stem tissue; activity was measured both before and after electrophoretic separation. The relationship of isoenzyme activity to gross (prior to electrophoretic separation) activity was investigated, as well as the relative behaviour of isoenzyme activity in the various genotypes and generations. Gross activity was correlated with isoenzyme activity; there was also evidence of maternal as well as genetic effects on isoenzyme activity.

1. Introduction

In a variety of plants, peroxidase has been shown, through the use of electrophoretic techniques, to consist of a number of isoenzymes. Shannon, Kay and Lew (1966), for example, isolated 7 peroxidase isoenzymes from roots of *Amoracia rusticana* (Horseradish); after purification, each isoenzyme was characterised on the basis of chromatographic behaviour, electrophoretic migration, spectrophotometric properties, and amino acid and carbohydrate composition. Andreev and Shaw (1965) found 7 anodal peroxidase isoenzymes in a cultivar of *Linum usitatissimum L.* (Flax) by using disc electrophoresis with acrylamide gels.

The results reported here were obtained from investigations of 2 *Linum usitatissimum L.* genotypes and their F_1, F_2 and first backcross progeny. Using acrylamide gels it was possible to separate 4 anodal isoenzymes from homogenates of main stem tissue, and to measure the activity of each of these isoenzymes in terms of areas obtained from densitometric scans of the gels. It was also possible to correlate the areas thus obtained with the measurements of 'gross' (in the sense of total isoenzymic) activity which were carried out on homogenates prior to electrophoretic separation.

2. Materials and Methods

The 2 genotypes of *Linum* used were the cultivars Royal (R) and Mandarin (M). These genotypes, and this species of *Linum*, are normally completely inbreeding. Crosses were made in all combinations between these 2 genotypes and their reciprocal F_1 hybrids; the crosses are shown schematically in table 1. The progeny resulting from the crosses contained the parental genotypes, their F_1's, plus the F_2's and first backcrosses. Plants from each of the 16 types of progeny shown in table 1 were raised in a growth chamber under conditions of light intensity, day length, temperature and humidity detailed in table 2. The plants were grown individually in pots in a soil/peat moss/sand mixture of ratio (by volume) 3:2:1, and were assayed at 70 days after germination. The plants of the various progeny types were allocated completely at random to positions within the growth chamber and at regular intervals during growth were rerandomised in position. The number of plants grown of each of the 16 types varied slightly; there were 8 plants each of types 11 and 44 (key as in table 1), 4 plants each of 14 and 41, and 9 plants each of all remaining (twelve) types. At 70 days the plants were cut at soil level, weighed fresh, and the leaves were removed from the centre (main) stem. The stems, extending from just above the cotyledons to just below the apical bud, were then homogenised in distilled water; the ratio of plant material to distilled water was constant. Homogenates (pH 5.5) were dialysed against distilled water to remove inhibitors, centrifuged at 1470 × g for 3 minutes, and stored at −30 °C. These active extracts of individual plants were assayed for gross peroxidase activity by the method of Maehly and Chance (1954), with the modifications detailed by Tyson and Jui (1967). The technique essentially involved following the rate of oxidation of guaiacol, after the addition of substrate, spectrophotometrically at a wavelength of 470 μm in the system shown below:

Table 1. *Key to products of crosses in all combinations between parental and F_1 genotypes*

Genotype:	Code number:
$R \times R$ = parent 1	1
$R \times M$ = F_1	2
$M \times R$ = F_1	3
$M \times M$ = parent	4

		Male parents: 1	2	3	4
Female parents:	1	11	12	13	14
	2	21	22	23	24
	3	31	32	33	34
	4	41	42	43	44

In the progeny of 1 to 4 crossed in all combinations:
11 and 44 = parental genotypes R and M
14 and 41 = F_1's
22, 23, 32, 33 = F_2's
12, 13, 21, 31 = backcrosses to R
24, 34, 42, 43 = backcrosses to M

Component	Ratio of components by volume:
10 mM phosphate buffer, pH 7.0	12.0
20 mM guaiacol	12.0
10 mM hydrogen peroxide	1.0

Table 2. *Growth chamber lighting, temperature and humidity regimes*

Hour	Light Intensity[1]	Temperature °C	Relative Humidity %
1 to 5	6000	22	30
6	6000	21	50
7	6000	20	55
8	6000	19	60
9	4000	18	60
10	4000	17	70
11	2000	16	75
12	2000	15	75
13 to 16	0	14	80
17	2000	14	75
18	2000	15	75
19	4000	16	65
20	4000	18	55
21	6000	20	45
22 to 24	6000	22	30

[1] Average values in foot candles under light rack at plant apex.

The readings were obtained on a Zeiss PMQ 11 spectrophotometer equipped with an automatic sample changer and a recorder. Peroxidase activities were expressed as the rates of increase in optical density (O.D.) per minute per unit of fresh weight through the calculation of the linear regressions of O.D. on time.

Before electrophoretic separations were carried out on the same homogenates which supplied the data for gross activity, these homogenates were concentrated with a polyacrylamide hydrogel. Electrophoretic separations of the active extracts were carried out in 7% acrylamide gels using a vertical electrophoresis cell of the type described by Jordan and Raymond (1967). The gel was prepared in a tris-(hydroxymethyl)-methylamine buffer at pH 8.9, while the electrode buffer was a tris-glycine system with a pH of 8.3. The buffers' constitutions were as follows:

	Tris	HCl (conc.)	Glycine	Distilled Water
Gel buffer	92.0 gm	approx. 8 ml.	—	Total vol. = 2 l.
Electrode buffer	1.2 gm	—	5.8 gm	Total vol. = 2 l.

The furthest point of migration in this anionic system was marked by the inclusion of traces of bromo-phenol blue in the samples. Prior to insertion of the samples into the cell, a constant voltage of 200 v. was applied to the gel for 30 minutes in order to remove impurities which otherwise affected the subsequent scanning of the gel tracks. A given sample was run in duplicate in 2 adjacent gel tracks; blank pockets in each gel provided a control. Migration of the samples took place at a constant voltage of 200 v. over the first 2 cms.; after the boundary had reached this point, the voltage was increased to a constant 400 v. until the boundary was 10 cms. from the start. After migration was complete, the gel tracks were incubated for 30 minutes at 22 °C. in the same reaction system used for the determination of gross activity. The reaction was stopped by transferring the gel tracks to 7% acetic acid for 30 minutes at the same temperature, before finally holding them in distilled water at 0–3 °C until scanned to obtain densitometric recordings of peroxidase activity along each gel track. Two problems were initially associated with electrophoretic separation and scanning. These were horizontal and vertical diffusion of stain, and fading of the colour which had developed during incubation. Staining of the complete gel slab allowed horizontal diffusion of stain across the closely adjacent gel tracks to occur. The difficulty was circumvented by casting the gel within the cell on a Perspex plate having vertical ribs on it. This plate remained in position throughout electrophoresis, effectively dividing the gel into 24 parallel tracks between which no horizontal diffusion could occur during the migration of proteins. On removal of the plate holding the gel from the cell, the individual gel tracks were readily extracted and then stained. Comparisons of the activity of a particular isoenzyme from one track, or sample, to the next were thus made feasible. Vertical stain diffusion, in terms of the areas of peaks on densitometric scans of adjacent isoenzymes on the same track was found to be minimal; the increase in area due to diffusion between the 2 adjacent isoenzymes most markedly different in activity was less than 2%. The activity of the more active isoenzyme of this pair exceeded, with very few exceptions, the level found for any isoenzyme in any of the samples of this study.

Gradual fading of the visible bands of colour of the gel tracks occurred after halting the reaction. Examination of the reaction involving active enzyme extract, buffer, guaiacol and hydrogen peroxide in terms of absorption spectra of this system in a cuvette at repeated, regular time points showed that the development of the colour in the visible range was correlated with an increase in the O.D. readings in the UV range of the spectrum. At 295 μm, decline in O.D. values was negligible in the cuvette system after extended periods from the start of the reaction; this was also true for gel tracks following halting of the reaction and holding at 0–3 °C. A wavelength of 295 μm was, therefore, used for scanning the gel tracks; the scanning was carried out with a Zeiss PMQ 11 spectrophotometer equipped with a CA 2 chromatogram attachment (linear transporter) and a recorder for the output of percentage transmission data. The data for gross peroxidase activity correlated well with the total activity of all isoenzymes.

Electrophoresis revealed 4 bands, corresponding to peroxidase activity, on the gel tracks. These isoenzymes were numbered from 1 to 4 in order of increasing relative mobility. Variation in relative mobility of a particular isoenzyme among individuals and progeny types was extremely small in comparison to the average differences in mobility between successive isoenzymes.

3. Results

Data on gross peroxidase activity from previous experiments (Tyson, 1969, 1970) revealed significant correlations between the means and variances calculated from individual plant data within each progeny type. These correlations were effectively removed by transforming the data to log values. The same type of correlation in the gross activity data here indicated, again, the need for transformation before further analysis; all gross activities were, accordingly, changed to log values. The data on isoenzyme activities were also transformed to log values in order to make the relationship between area and concentration a linear one. Examination, after transformation, of the within-progeny-type correlations between means and variances for each isoenzyme showed that none of them was significant. Table 3 contains the gross activities for each of the 16 progeny

Table 3. *Gross peroxidase activity and activities of each of the 4 peroxidase isoenzymes. Means shown for each type of progeny resulting from crossing scheme of Table 1*

Progeny type	Gross activity	Activity of isoenzyme 1	2	3	4	Total activity of all isoenzymes
11	0.055	1.50	0.82	1.15	2.67	6.14
12	0.053	1.73	1.56	0.94	2.82	7.05
13	0.059	1.50	0.93	0.78	2.33	5.54
14	0.043	1.28	0.82	0.81	2.66	5.57
21	0.054	1.35	0.86	0.81	2.84	5.86
22	0.045	1.40	0.70	0.83	2.84	5.77
23	0.052	1.04	0.41	0.48	2.68	4.61
24	0.045	1.33	0.85	0.93	2.76	5.87
31	0.060	1.33	0.50	0.93	2.77	5.53
32	0.049	1.51	1.18	1.06	2.82	6.57
33	0.054	1.22	0.70	0.71	2.79	5.42
34	0.058	1.42	0.59	1.10	3.03	6.14
41	0.029	1.16	0.80	0.31	2.64	4.91
42	0.048	1.38	0.0	1.05	2.99	5.42
43	0.035	0.70	0.09	0.54	2.82	4.15
44	0.034	1.27	0.66	0.78	2.84	5.55
Relative Mobility of Isoenzyme:		0.07		0.13	0.20	0.60
Percent Contribution to Total Isoenzyme Activity:		23.5%	12.7%	14.7%	49.1%	

types, together with the corresponding isoenzyme activities and the relative mobility, averaged over all progeny types, for each isoenzyme.

The correlation between gross peroxidase activities and the sum total of the isoenzymes' activities was examined over the 16 progeny types. Although the relationship was positive, it was not significant, ($r = 0.41$). There were, however, a number of plants within each progeny type, and the possible relationship of gross to isoenzyme activity was pursued on the level of variation between individual plants within progeny types. The multiple linear regression of gross activity on the activities of isoenzymes 1, 2, 3 and 4 was calculated from within-progeny-type sums of squares and cross products. Of the (132-1) degrees of freedom available, 15 were accounted for by differences between progeny types, leaving 116 within progeny types. These sums of squares and cross products, calculated within each of the progeny types, were then pooled, and the 5 parameters, b_0 to b_4, estimated. The resultant regression accounted for a significant portion of the variation in Y, gross peroxidase activity; the analysis of variance of the regression is shown in table 4. Since the 16 progeny types contained non-segregating as well as segregating generations, appropriate pooling of sums of squares and cross products allowed the extraction of regressions separately for these 2 main groups. Regressions could be calculated in the situation where plant-to-plant variability was purely environmental, in contrast to the case where such variability was partly genetic and partly environmental. Thus progeny types 11, 14, 41 and 44 supplied the statistics for the non-segregating generations, while the remaining (twelve) types supplied those for segregating generations. From this latter group it was also possible to extract regressions for F_2's as distinct from backcrosses to parent 1 or to parent 2. Pooling from 22, 23, 32 and 33 supplied the F_2 regression, while pooling from 12, 13, 21 and 31, or from 24, 34, 42, and 43 provided each of the backcross regressions. The analyses of variance of these further regressions are also shown in table 4. The results from the regressions, in terms of partial regression coefficients, are shown in table 5. Elimination of X (isoenzyme) data which yielded partial regression coefficients not significantly different from zero resulted in the coefficients shown in table 6. In each of the backcrosses, as well as in the non-segregating, generations, only one of the isoenzymes predicted gross activity. In the F_2, none of the partial regression coefficients was significant; the simple correlations between the X variables were uniformly high here. The significant partial regressions in table 6 suggested that the particular isoenzyme predicting gross activity varied with the generation. In the non-segregating generations, isoenzyme 4 predicted gross activity, but it was not possible to exclude heterogeneity in this amongst the 4 progeny types (11, 14, 41, 44) pooled to obtain this result. The data in these 4 progeny types were not extensive enough to estimate b_0 to b_4 within each of them separately. Nevertheless, examination of

Table 4. *Multiple linear regressions of gross activity on activities of isoenzymes 1, 2, 3 and 4. Analyses of variance*

Item		*DF*	mean square	*F*
All Progeny Types, 11 to 44	Regression	4	0.00267560	18.12***
	Residual	112	0.00014767	
Non Segregating 11, 14, 41, 44	Regression	4	0.00054491	6.53**
	Residual	16	0.00008347	
F_2's only 22, 23, 32, 33	Regression	4	0.00047568	3.71*
	Residual	28	0.00012821	
Backcross 1's only 12, 13, 21, 31	Regression	4	0.00116408	7.37**
	Residual	28	0.00015790	
Backcross 2's only 24, 34, 42, 43	Regression	4	0.00080234	3.78*
	Residual	28	0.00021240	

* Significant at Probability 0.05.
** Significant at Probability 0.01.
*** Significant at Probability 0.001.

Table 5. *Multiple linear regressions of gross activity on isoenzyme activity calculated for*

Partial[1] Regression Coefficients	All Progeny Types, 11 to 44	Non Segregating Generations	F_2 Progeny	Backcross 1 Progeny	Backcross 2 Progeny
b_1	0.00588977	−0.00215771	0.00698292	−0.01960428	0.02318495*
	±0.00519615	±0.01130707	±0.01006179	±0.01408612	±0.01133501
b_2	0.00098639	−0.00151794	0.00131885	0.01660331*	−0.00495494
	±0.00232594	±0.00397492	±0.00488791	±0.00648875	±0.00499467
b_3	0.02321777***	0.01019149	0.01184102	0.05076982***	−0.00247734
	±0.00520192	±0.01055035	±0.01440659	±0.01224023	±0.01474473
b_4	−0.00759372	0.02944052*	0.00727080	−0.01058909	0.02322834
	±0.00388973	±0.01131282	±0.01789941	±0.00636209	±0.01781394

[1] Where b_1 indicates the coefficient for isoenzyme No. 1, and so on.

* Significant at Probability 0.05. *** Significant at Probability 0.001.

Table 6. *Linear regression coefficients calculated for*

Non-Segregating Generations:	Backcross 1 Progeny	Backcross 2 Progeny
$b_4 = 0.03823668 \pm 0.00749933$	$b_3 = 0.02296738 \pm 0.00716589$	$b_1 = 0.02827847 \pm 0.00451552$

the simple correlation coefficients (r) between gross activity and the activity of each of the isoenzymes within each of these progeny types, followed by ranking of the r values from highest to lowest, suggested that there was some agreement among 11, 14, 41 and 44. The results of the ranking are shown below:

	Progeny type:			
	11	41	41	44
r values: 1st.	3	4	4	4
2nd.	4	1	1	3
3rd.	1	2	3	1
4th.	2	3	2	2

The numbers in the table indicate a particular isoenzyme; in 3 of the 4 progeny types, gross activity was correlated most highly with isoenzyme 4 activity. In the case of backcross 1, isoenzyme 3 showed gross activity-predictive variability; for backcross 2, it was isoenzyme 1.

An analysis of variance of the isoenzyme activities was carried out; progeny type and isoenzyme effects were regarded as fixed. Since the $(16 \times 4) = 64$ subclasses did not contain a uniform number of observations, the model was fitted to the data by a least squares procedure of the type described by Draper and Smith (1968). The results of the analysis of variance are shown in table 7; there was a significant interaction between progeny types and isoenzymes. The interaction sum of squares was then broken down in order to determine the source of the interaction. The orthogonal comparisons utilised in this breakdown are shown in table 8 in terms of suitable coefficients. A transformation matrix was obtained from

Table 7. *Analysis of variance of isoenzyme activity*

Item	*DF*	Mean Square	*F*
Progeny Type (*P*)	15	0.56269045	4.85***
Isoenzyme (*I*)	3	75.72507069	652.49***
$P \times I$ Interaction	45	0.21172099	1.82**
Error	464	0.11605516	

** Significant at Probability 0.01.
*** Significant at Probability 0.001.

Table 8. *Orthogonal breakdown of the 15 degrees of freedom for progeny types*

Comparison:	Progeny Types 11	12	13	14	21	22	23	24	31	32	33	34	41	42	43	44
1	1															−1
2				1									−1			
3	1			−1									−1			1
4						1	1			−1	−1					
5						1	−1			1	−1					
6						1	−1			−1	1					
7		1	1		−1				−1							
8		1	−1		1				−1							
9		1	−1		−1				1							
10								1				1		−1	−1	
11								1				−1		1	−1	
12								1				−1		−1	1	
13		1	1		1			−1	1			−1		−1	−1	
14	1			1		−1	−1			−1	−1		1			1
15	1	−1	−1	1	−1	1	1	−1	−1	1	1	−1	1	−1	−1	1

Orthogonal breakdown of the 3 degrees of freedom for isoenzymes

Comparison	Isoenzyme: 1	2	3	4
1	1	−1		
2	1	1	−2	
3	1	1	1	−3

these coefficients to effect the single degree of freedom orthogonal contrasts in the way outlined by Harvey (1968). The sums of squares for each of these contrasts are shown in table 9. Each of the 15 comparisons shown in the first part of table 8 was applied to the 3 comparisons, relating to isoenzymes, in the second part. There were, therefore, 15 sets of 3 contrasts in the interaction; contrasts 1, 2 and 3 in table 9 represent comparison 1 among progeny types applied to comparisons 1, 2, and 3 among isoenzymes, and so on. The sums of squares for each of the 45 contrasts in the interaction were individually tested against the error mean square. From table 9 it can be seen that there were significant interactions in backcrosses 1, 2, and in the comparison of the mean of backcross 1's to the mean of backcross 2's. In the backcross 1's the comparison made was between the use of parent 1 as a female versus its use as a male in crosses to the F_1; it appeared that the relationship between isoenzyme 3 with 1 and 2, and between isoenzyme 4 with 1, 2 and 3, changed depending on this. Explanation of the interactions involving backcross 2 may be derived in a similar fashion from tables 8 and 9. The 2 remaining significant interactions were concerned with the $B_1 - B_2$ difference (contrasts 38 and 39). There was no change in the relationship of isoenzyme 1 to 2, but the relationship of 3 to 1 and 2, and of 4 to 1, 2 and 3 changed between backcrosses 1 and 2. The interaction of isoenzymes 1 and 3 with the parent used in generating the backcross paralleled the regression results. In the comparison of the 2 parental genotypes (contrast 3) the change in the relationship of isoenzyme 4 to 1, 2 and 3 approached significance; this paralleled the suggestion, from ranking of correlation coefficients for non-segregating generations, that isoenzymes 3 and 4 behaved differently in the 2 parents.

Table 9. *Breakdown of progeny type by isoenzyme interaction into single degree of freedom contrasts*

Progeny type Comparison	Isoenzyme Comparison	Contrast	Sum of Squares	Progeny type Comparison	Isoenzyme Comparison	Contrast	Sum of Squares
1	1	1	0.01145266	9	1	25	0.01048873
	2	2	0.04844960		2	26	0.00018091
	3	3	0.41403485		3	27	0.02344337
2	1	4	0.00547717	10	1	28	0.17829768
	2	5	0.08623116		2	29	0.22762848
	3	6	0.02181903		3	30	0.58333356*
3	1	7	0.05920818	11	1	31	0.12420380
	2	8	0.16800677		2	32	0.00070113
	3	9	0.00559896		3	33	0.21838623
4	1	10	0.08569997	12	1	34	0.68181675*
	2	11	0.00363178		2	35	0.12179136
	3	12	0.10753613		3	36	0.03132993
5	1	13	0.00297608	13	1	37	0.23969366
	2	14	0.00416095		2	38	1.32111451***
	3	15	0.20309466		3	39	1.74982068***
6	1	16	0.3830836	14	1	40	0.00031513
	2	17	0.00190788		2	41	0.00003136
	3	18	0.00387171		3	42	0.07176054
7	1	19	0.12724002	15	1	43	0.12011952
	2	20	0.68329092*		2	44	0.11646202
	3	21	1.02513486*		3	45	0.02571866
8	1	22	0.29962175			Total:	9.56089615
	2	23	0.24313098				
	3	24	0.02949773				

* Significant at Probability 0.05.
*** Significant at Probability 0.001.

4. Discussion

The progeny types used in this study formed part (replicate 1) of a larger investigation of gross peroxidase activity, the results of which have been reported (Tyson, 1970). The progeny type means for gross activity here and the corresponding means from

replicates 2 or 3 of this larger investigation were positively and significantly correlated. The results of measurements of isoenzyme activity, which have been described here, suggested that gross peroxidase activity was related to the activity of the peroxidase isoenzymes.

The calculation of multiple linear regressions to obtain the relationship between gross activity and individual isoenzyme activity took no account of possible errors in measurements of isoenzyme activity, or, in the extraction of separate regressions for different generations, of possible heterogeneity within the F_2's or backcrosses. The analysis of variance of the isoenzymes' data indicated that in the F_2 generation the comparisons made among them were unaffected by the 4 ways in which this generation was produced. This was not true, however, for either of the backcrosses; here there was, for example, evidence of reciprocal effects on the behaviour of the isoenzymes relative to one another. Such effects must presumably have been involved in the disparity detected in the relative behaviour of the isoenzymes between the 2 groups of backcrosses to each parent (contrasts 38, 39). This heterogeneity within each backcross group also made suspect the simple switch from isoenzyme 3 in backcross 1 to isoenzyme 1 in backcross 2 as the predictor of gross activity. For the non-segregating generations, the near significance of contrast 3 suggested that there was a parental difference in the relative behaviour of the isoenzymes; this paralleled the change in ranking of the simple correlation coefficients relating individual isoenzyme activity to gross activity for each parent. From the variation of isoenzyme 4 in 3 of the non-segregating genotypes, the situation changed in the F_2 to one where the 4 isoenzymes varied together essentially as a unit. There was evidence, therefore, of genetic as well as maternal effects on the relative behaviour of the 4 isoenzymes.

Acknowledgements

The work reported here was supported by a grant from the National Research Council of Canada, to whom thanks are extended. Thanks are also extended to Miss Edith Chan and Mr. Harvey Nickerson for their technical assistance during the course of this work.

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Received October 13, 1970

Communicated by R. Hagemann

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