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Distinguishing Rose Cultivars by Polyacrylamide Gel Electrophoresis. I. Extraction and Storage of Protein and Active Enzymes from Rose Leaves[1]

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Abstract. **Procedures were developed to extract protein and active enzymes from rose leaves. A suitable extraction medium, extraction time, and sample application rate was determined for each system in which bands could be separated. Sixteen enzyme systems and anionic protein were investigated, with only anionic protein, peroxidase, esterase, malate dehydrogenase, cytochrome oxidase, phenoloxidase, and polyphenoloxidase producing clear bands. Existing staining techniques were adequate for staining cytochrome oxidase, phenoloxidase, and polyphenoloxidase activity, but modifications of the reported anionic protein, peroxidase, esterase and malate dehydrogenase stains were required. Isoenzyme changes which occur during 0, 9, 12, and 16 weeks storage are discussed.**

Preparation of a protein sample for electrophoresis is complicated by the interactions that can occur among proteins and other cellular constituents that are rigidly compartmentalized *in vivo,* but are mixed during tissue maceration, including vacuolar acids, carbohydrates, hydrolytic and oxidative enzymes, phytic acid, and phenolic compounds (26). The objective of this study was to develop a technique to extract active enzymes from rose leaves with minimal interference from the degradative constituents.

With some plants, crude extracts can be used with no adverse effects on results (6, 17, 32); while with others, protective measures must be taken (12, 18). The major interaction to be controlled is that between proteins and phenols and their oxidation products (1, 11, 17, 21). Phenols form complexes with proteins through strong H-bonds and are readily oxidized to quinones which in turn oxidize essential groups of proteins or form covalent bonds to the protein (21). Roses have been found to have an exceptionally high phenolic content (17).

Many methods have been developed to prevent protein-phenol interaction during the extraction process, with no single extraction technique effective for all enzymes of a species or for the extraction of a particular enzyme from all plant species (12). The choice of extraction technique and solution has been found to affect final results significantly (6). Dimethyl sulfoxide (DMSO) is an organic solvent in which enzymes maintain activity for prolonged periods (12-20 hours) at moderate temp (37°C) in high solvent concn (35-60%) (23). It has been used as an extraction medium for soluble plant proteins and offers the advantages of simplicity, room temp operation, and a storable produce (2). Extraction of leaves with borate yield samples with high total protein and enzyme activity, but was effective only in an extremely narrow pH range (19). Reducing agents such as ascorbate, potassium metabisulfite ($K_2S_2O_5$), cysteine, mercaptoethanol, and dithiothreitol (DTT) prevent the oxidation of phenols and the accumulation of quinones, thus lowering the probability they will react with proteins (16, 21). Extraction with reducing agents alone yields satisfactory results in some cases (29), but much more rigorous procedures are required in others (11, 16, 28). The use of reducing agents sometimes causes protein modification (21) or interference during electrophoresis (28).

In some studies merely preventing the oxidation of phenols does little good; it is necessary to remove them (11, 17, 21). Techniques which separate proteins and phenols include dialysis, gel filtration, the use of polyvinylpyrrolidone (PVP) (1, 11, 13, 17, 21), and the preparation of acetone powders (3, 16). Dialysis and gel filtration work only if adequate precautions are taken to prevent oxidation, as oxidation products will not separate from the proteins (21). If the phenolic content of the sample is sufficiently high, neither dialysis nor gel filtration will separate the proteins and phenols fast enough to prevent enzyme inactiviation (11).

Insoluble PVP contains groups similar to the peptide linkage of proteins. If excess PVP is added to the sample, those phenols which form H-bonded complexes with protein will bind instead to the PVP and can be removed by centrifugation (10, 21). Samples can also be passed through a column of PVP to remove phenols (13). Denaturing agents such as urea should not be included when extracting with PVP because they cause the PVP-phenol complex to dissociate (1). Again, if the phenolic concn of the sample is sufficiently high, the use of PVP alone will not remove all the phenols and some enzyme inactivation may still occur (11, 18).

Acetone can be used to precipitate protein with little loss of enzyme activity and at the same time extract most phenols (3). Bendall and Gregory (3) recommended 20% water be included in the acetone except for a final drying of the precipitate with pure acetone, as phospholipids are soluble in aqueous but not dry acetone, and some enzymes are tightly bound as lipoprotein particles. Hare (16) recommended including 30% water to remove water soluble interfering substances such as chlorophyll. Dirr et al. (11) found the soluble fraction of acetone preparations to be inhibitory to enzyme activity while the precipitate contained no inhibitors. Though the preparation of acetone powders separates most phenols and enzyme inhibitors from the protein fraction, some combinations of PVP and/or reducing agents are still necessary when resolubilizing the protein to prevent enzyme inactivation (16, 28).

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Other frequently used components of an extraction solution include sucrose, urea, detergents, and salts for increasing ionic strength. Sucrose enhances chloroplast disruption by its influence on osomotic pressure, and minimizes the aggregation and precipitation of leaf proteins after they are extracted (4). Urea increases the solubility of certain classes of proteins and the number of detectable isoenzymes of some systems by causing disassociation of H-bonds (14, 16). Since removal of urea may lead to reaggregation, samples containing urea should be run on gels containing urea. Both sucrose and urea increase sample density sufficiently to preclude the need for a sample gel. Detergents such as Triton X-100, Tween-20, or Tween-80 also increase the solubility of some proteins (4, 16).

Techniques for preparing tissue for maceration vary considerably. The tissue may be frozen at -2^{o} to -20^{o}C (21), in liquid nitrogen (LN) (7, 23), lyophilized (7), or simply ground fresh (29). Bonner et al. (5) found no difference in peroxidase zymograms between tissues used directly and tissue that were frozen at -2^{o} to -4^{o} prior to use. Betschart and Kinsella (4) observed that lyophilization did not impair protein extractability of alfalfa leaves, but it did reduce protein yield from soybean leaves. Freezing in LN facilitates tissue maceration and effectively stops all interactions between cell components.

Samples may be stored as frozen (5, 30) or lyophilized (18, 25) tissue, acetone powders (16, 22), or frozen solutions (27). Length of storage and storage conditions are dependent on the enzymes to be analyzed.

Materials and Methods

While determining the usefulness of an extraction procedure, samples were run on polyacrylamide gels as by Davis (9), with the exception that no sample gel was used. The density of samples was increased by making them 15% sucrose. Gels were run at 3 ma/tube and stained for total protein (9) and peroxidase (24). The no. and clarity of bands obtained was used to compare extraction techniques.

Immediately after removal from the plant, rose leaves were prepared for extraction by thoroughly washing them in cold water containing a detergent, rinsing in distilled water, and blotting dry. Five extraction techniques were evaluated and included:

1. Extraction in 30% DMSO as described by Ascher and Weinheimer (2);
2. Preparation of acetone powders as described by Hare (16);
3. Preparation of acetone powders in a 2°C refrigerated room; and
4. Freezing leaves in LN, grinding in a LN cooled mortar and pestle, homogenization with an extraction solution in a Potter-Elvehjem homogenizer, and centrifugation.

The 5th method was a combination of the 2nd and 4th. Prepared leaves were placed in LN and ground in a LN cooled mortar and pestle. The resultant powder was homogenized for 30 sec in a Potter-Elvehjem homogenizer with 5 parts 70% acetone (all acetone used in the extraction process was maintained at -20^{o}C). The 30 sec homogenization was repeated twice, with the mortar cooled in an ice water bath 1 min between each grinding. The slurry was filtered under suction, and the residue was washed with 100% acetone until the filtrate was clear. Excess acetone was drawn off under suction, and the residue was lyophilized (Fig. 1).

To determine the need for the components of extraction solutions used by others (16, R. G. McDaniel, personal communication), the following solutions were prepared and used to extract the protein from rose acetone powders and commercially prepared bovine serum albumen (BSA) and horseradish peroxidase (HRP).

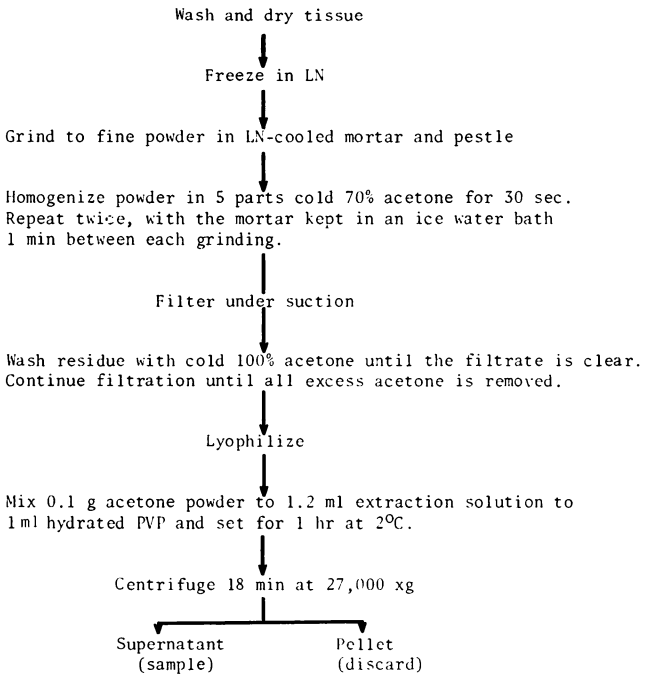

Fig. 1. Flow diagram for extraction and preparation of acetone powders from rose leaves.

A.	DTT	0.05 g
	$K_2S_2O_5$	0.25 g
	Ascorbic acid	0.50 g
	PVP	0.75 g
	Sucrose	7.50 g
	Triton X-100	2 ml of 10%
	Tris	pH to 7.3
	Water	to 50 ml
B.	PVP	0.75 g
	Sucrose	7.50 g
	Triton X-100	2 ml of 10%
	0.1 M Na phosphate buffer, pH 7.3	to 50 ml
C.	Sucrose	7.50 g
	0.1 M Na phosphate buffer, pH 7.3	to 50 ml

Extracting with urea (14, 16) and/or a high ionic strength solution (6, 30, G. Chism, personal communication) has been shown to improve the extraction of some enzymes. In this study, where urea was used, it was added to the supernatant fluid following separation of the PVP by centrifugation to prevent dissociation of the PVP-phenol complex. To increase the ionic strength of the solution, 0.5 M $CaCl_2$ was used. Its addition to the complete extraction solution caused precipitation of an undetermined compound. By stepwise addition of the individual components, $K_2S_2O_5$ was found to be causing the precipitation and was eliminated whenever $CaCl_2$ was used. A factorial experiment using all combinations of + or −15% urea and + or −0.5 M $CaCl_2$ was run for each enzyme studied to determine which combination produced the best results for each particular enzyme. When urea was not included, solutions were made to 15% sucrose.

Several tissue:extraction solution ratios were evaluated. Preliminary data showed that a tissue:extraction solution ratio of 0.1 g acetone powder to 1.2 ml extraction solution to 1 ml hydrated PVP was satisfactory for all enzyme systems evaluated in this study. This ratio produced a sample which could be

applied to the gels without being concentrated.

Extraction times of 1 hr, 6 hr, and about 16 hr (overnight) were compared and found to produce similar results with peroxidase and total protein. The 1 hr extraction was used for the duration of the study, and samples were used immediately after preparation.

Sixteen enzyme systems which have been used in other electrophoretic studies of plant tissues, and anionic protein, were evaluated on their ability to yield clear, reproducible banding patterns. Several staining techniques were examined for most of the enzymes. The enzymes and staining techniques evaluated are listed in Table 1.

To determine the effects of storage on the systems which produced acceptable bands, lyophilized acetone powders stored at $-20^{\circ}C$ were extracted and run after 0, 9, 12 and 16 weeks of storage. In recording results, each band was identified by its R_f value and density.

$$R_f = \frac{\text{distance enzyme band migrated}}{\text{distance marker dye migrated}}$$

Density = light (L); medium (M); or heavy (H)

Natarella and Sink (22) found the marker dye completely disappeared in peroxidase stain solution so gels were cut with a razor blade at the marker dye prior to staining. Photographs were taken of all gels, but as reported by Hare (16) and Clements (8), seldom showed all visible bands. Therefore, zymograms were drawn based on the average R_f values and density recordings of at least 4 gels.

Ten of the enzyme systems evaluated were eliminated from the study because preliminary experiments indicated an absence of banding, or in the case of ascorbic acid oxidase, band instability. Of the 7 remaining systems, cytochrome oxidase, phenoloxidase and polyphenoloxidase were stained as described by Hare (16). The final esterase and malate dehydrogenase staining methods were combinations of those developed by Scandalios (24) and Hare (16).

Table 1. Results of staining procedure screenings for enzyme systems.

Enzyme	Stain reference	Results
Acid phosphatase	6, 15, 24	No staining; difficult to get substrate and stain into solution.
Alcohol dehydrogenase	16, 24	Enzyme present as a wide blur, no banding.
Alkaline phosphatase	6, 15, 24	No staining.
L-Amino acid oxidase	15, 16	No staining.
Alpha amylase	6, 15, 24	Enzyme present as a wide blur, no banding.
Anionic protein	9, 31	Good banding with considerable background staining.
Ascorbic acid oxidase	16	Good bands but fade too fast for consistent recording.
Catalase	6, 15, 16, 24	Enzyme present as a wide blur, no banding.
Cytochrome oxidase	16	Good banding.
Esterase	16, 24	Good banding.
Formazan oxidase	16	No staining.
Alpha ketoglutaric acid dehydrogenase	16	Enzyme present as a wide no banding.
Malate dehydrogenase	16, 24	Good banding.
Peroxidase	6, 15, 16, 20	Good banding.
Phenoloxidase	16	Good banding with some background staining.
Phosphorylase	15	No staining.
Polyphenoloxidase	15, 16	Good banding.

Esterase. After running, gels were placed in a pH 6.2, 0.1 M Na phosphate buffer for 5 min and then placed in the substrate solution at $37^{\circ}C$ until bands developed. The substrate solution contained 70 mg fast blue RR salt, dissolved in 100 ml buffer and filtered just prior to use, and 2 ml of alpha naphthyl acetate (1.0% in 1 acetone: 1 water), which was added after filtration. Stained gels were washed and fixed in 10% trichloracetic acid (TCA).

Malate dehydrogenase. The reagent for estimation of malate dehydrogenase activity was prepared by mixing equal portions of Solution A and Solution B stocks. Solution A: 25 mg nitroblue tetrazolium (NBT) (dissolved in a minimum amount of ethanol), 80 mg $MgSO_4 \cdot 7H_2O$, and 2 mg KCN dissolved in 40 ml tris-HCl buffer pH 7.5, 0.1 M. Solution B: 2.5 mg phenazine methosulfate (PMS) dissolved in 40 ml buffer. Just before use, the solutions were mixed in dim light, 0.584 g dl-malic acid (0.05 M) was added, the pH was adjusted to 7.5 with NAOH and 56 g nicotinamide adenine dinucleotide (NAD) was added (the pH must be adjusted before addition of the NAD). Gels were incubated in this solution in the dark at $37^{\circ}C$ until bands developed, then washed and fixed in 2% acetic acid.

The final peroxidase staining method consisted of incubating gels in a 0.5% benzidine and 0.03% H_2O_2 solution until bands were well developed, transferring them to 7% acetic acid for 5 min, and rinsing and holding in distilled water. The stain solution was prepared by dissolving 0.5 g benzidine in 4.5 ml of acetic acid, bringing it to 100 ml with demineralized soluble distilled water, and adding 0.1 ml 30% H_2O_2 just prior to use.

Coomassie brilliant blue proved to be the superior stain for anionic (total) protein. The staining solution used contained 0.025% coomassie brilliant blue, 10% acetic acid, and 25% methanol. After staining for 12 hr, gels were destained in 7.5% acetic acid and 50% methanol in a diffusion destainer, then rehydrated and stored in distilled water.

Results

Samples extracted in 30% DMSO produced no anionic (total) protein or peroxidase bands. Acetone powders prepared as described by Hare (16) yielded some bands, but they were few and diffuse. When this extraction process was moved to the refrigerated room good bands were obtained.

Extraction of the protein immediately after tissue maceration in LN was unsuitable because samples were too dilute for direct application to the gels. Concentrating samples with Millipore filters or dialysis bags in a hydroscopic compound (aquacide) proved to be unsuitable for the multi-enzyme sample obtained in this study. Also, long term storage of the macerated tissue or extracted sample was not possible without considerable enzyme alteration. Samples extracted from the acetone powders prepared from the tissue macerated in LN produced numerous distinct bands.

Commercially prepared samples of BSA and HRP extracted with solutions B and C yielded sharper bands than samples extracted with the solution containing the reducing agents. With the rose acetone powders no peroxidase bands developed, while anionic (total) protein bands were diffuse and hard to distinguish from a heavy background stain, unless reducing agents were included in the extraction solutions.

Different systems were best extracted with different combinations of $CaCl_2$ and urea in the extraction solution and required different extract application rates for optimum band clarity (Table 2 and Fig. 2).

Table 2. Optimum extraction solution and application rates for rose enzymes.

Enzyme	Optimum extraction solution	Optimum application rate (ml)
Cytochrome oxidase	$-C-U^z$	0.30
Esterase	$-C-U^z$	0.05
Malate dehydrogenase	$+C+U^y$	0.30
Peroxidase	$+C+U^y$	0.20
Phenoloxidase	$-C-U^z$	0.30
Polyphenoloxidase	$-C-U^z$	0.10
Total protein	$+C-U^x$	0.25

^z-C-U means $CaCl_2$ was not included in the extraction solution and 15% sucrose was added to the sample solution.

^y+C+U means $CaCl_2$ was included in the extraction solution and 15% urea was added to the sample solution.

^x+C-U means $CaCl_2$ was included in the extraction solution and 15% sucrose was added to the sample solution.

The stability in storage of the 7 enzyme systems varied. *Anionic protein.* After 9 weeks' storage, 1 light band disappeared, 3 medium bands faded to light, and the mobility of the fastest band decreased slightly. After 12 weeks storage another light band disappeared and after 16 weeks 4 more bands

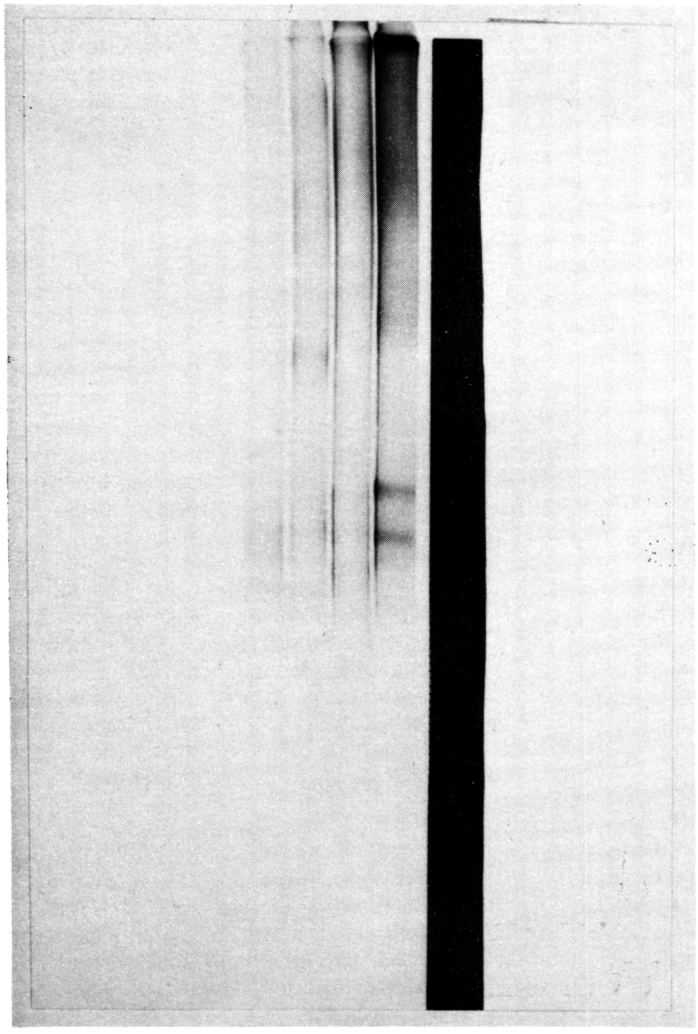

Fig. 2. Inhibition of esterase by $CaCl_2$ and/or urea. Sample extraction solutions were (left to right): +C+U, −C+U, +C−U, and −C−U with respect to $CaCl_2$ and urea. (See Table 2 for explanation.)

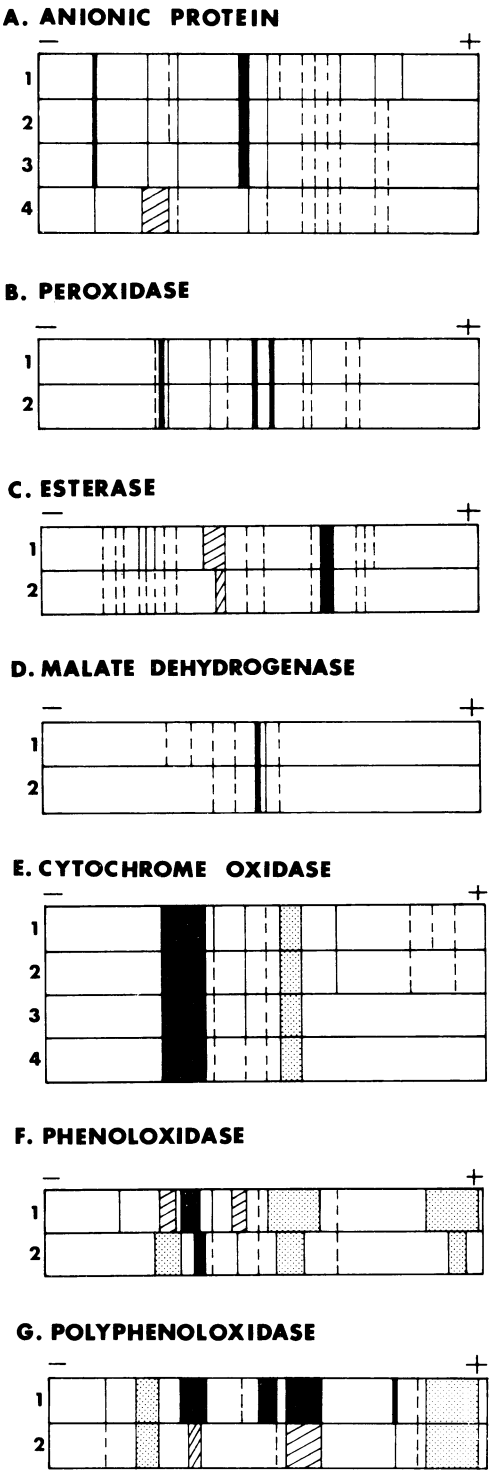

Fig. 3. Effects of storage at −20°C on the isoenzyme banding patterns of lyophilized acetone powders of rose leaves. A. Anionic protein banding patterns of 'Candy Stripe' rose leaves after 0[1], 9[2], 12[3], 16[4] weeks' storage. B. Peroxidase isoenzyme patterns of 'Peace' rose leaves after 0[1] and 16[2] weeks' storage. C. Esterase isoenzyme patterns of 'Candy Stripe' rose leaves after 0[1] and 12[2] weeks' storage. D. Malate dehydrogenase isoenzyme patterns of 'Candy Stripe' rose leaves after 0[1] and 16[2] weeks' storage. E. Cytochrome oxidase isoenzyme patterns of 'Peace' rose leaves after 0[1], 9[2], 12[3], 16[4] weeks' storage. F. Phenoloxidase isoenzyme patterns of 'Peace' rose leaves after 0[1] and 16[2] weeks' storage. G. Polyphenoloxidase isoenzyme patterns of 'Chicago Peace' rose leaves after 0[1] and 16[2] weeks' storage. In all cases, dashed lines and dotted areas indicate light stained bands; light solid lines indicate medium stained bands; and heavy shading indicates heavily stained bands.

faded 1 density level, and another changed from sharp to blurred (Fig. 3).

Peroxidase. Peroxidase was extremely stable in storage, and even after 16 weeks the only change recorded was 1 medium band faded to light (Fig. 3).

Esterase. After 12 weeks' storage, 1 light band faded and 3 medium bands faded to light (Fig. 3). Data for 9 and 16 weeks are unavailable.

Malate dehydrogenase. Two light bands disappeared during the first 9 weeks of storage, but all others were stable with respect to both mobility and density for at least 16 weeks (Fig. 3).

Cytochrome oxidase. After 9 weeks' storage, 1 light band disappeared; and after 12 weeks 2 additional light bands and 1 medium band disappeared (Fig. 3).

Phenoloxidase. The only change after 9 weeks' storage was in the density of 1 band from medium to light. After 12 weeks the bank disappeared and 1 additional band changed from medium to light. There were no other changes after 16 weeks' storage (Fig. 3).

Polyphenoloxidase. There was a loss of 1 light band and a density decrease in 5 bands after 9 week's storage, but no more changes occurred up to 16 weeks (Fig. 3).

Discussion

Extraction with DMSO or preparation of acetone powders outside the refrigerated room did not produce samples which would yield clear bands. That acetone powders prepared in the refrigerated room produced clear bands indicates continuous temp control during the extraction process is essential. Freezing tissue in LN prior to preparation of acetone powders proved to be effective in minimizing the temp dependent reactions which interfered with enzyme activity. Both methods resulted in satisfactory banding but the LN method was used throughout the remainder of the experiment to avoid the possibility of protein hydrolysis by proteases which might be active at high subzero temps.

As in other studies (21, 28), reducing agents interfered with the clarity of bands obtained following electrophoresis. Despite the interference, the reducing agents were required for this study because of the exceedingly high phenolic content of roses (17). Apparently, even the rigorous acetone powder preparatory procedures do not remove all of the phenols, so the reducing agents are needed to prevent oxidation of the remaining phenols to quinones, which irreversibly bind to proteins.

Specific extraction procedures must be determined for each enzyme system to be studied. Results with catalase were poor in this study probably because samples to be examined for catalase activity should not be lyophilized. The components of the optimum extraction solution must also be determined. A solution with ionic strength and containing a dissociating agent was necessary to obtain maximum band numbers and clarity with malate dehydrogenase and peroxidase, while it interfered with the extraction or activity of esterase, cytochrome oxidase, phenoloxidase and polyphenoloxidase. The H-bond dissociation caused by 15% urea apparently separates some enzymes into active subunits. Future studies should include a range of urea concn. Lower concn may produce active subunits of esterase, cytochrome oxidase, phenoloxidase, and polyphenoloxidase and higher concn may yield additional active subunits of malate dehydrogenase and peroxidase. Salts other than $CaCl_2$ and concn other than 0.5 M may produce better extraction results than obtained in this study and should be evaluated. Determining the optimum application rate is important because application of too much sample results in blurred bands and too little in missing bands.

Satisfactory bands may be obtained with the first staining technique tried, but in many cases several techniques must be examined before results will be acceptable. The esterase, malate dehydrogenase, peroxidase and anionic (total) protein stains used in this study were all combinations of methods developed for other species. Factors that were varied when evaluating the stains were pH, type of buffer, buffer strength, substrate, stain, and activating ions. By determining the proper extraction technique and extraction and staining solution, the 10 enzyme systems which did not produce bands might be utilized in future studies.

Though there were few qualitative changes after 12 weeks' storage, samples to be stained for anionic protein should be compared only with other samples stored the same length of time under similar conditions because of the possible loss of light bands during storage. If the relative density of individual bands is to be accurately measured for use in distinguishing cultivars, fresh material should be used because of the fading.

The stability of peroxidase in this study suggests fresh samples can be compared with stored samples and the density of isoenzymes of stored samples can be quantified and used in comparisons. Liu (20) has reported that the electrophoretic mobility of peroxidase isoenzymes can be altered considerably with no loss of activity by storage at 4°C at pH 7.0 or higher. Apparently, the lyophilized acetone powder is a more stable storage form for peroxidase.

As with total protein, samples to be stained for esterase can be stored for 12 weeks, but stored samples should be compared with those stored an equal length of time, and fresh samples should be used if bands are to be quantified by densitometry.

With malate dehydrogenase, the instability of the 2 lost bands relative to the stability of the others suggests they should not be used in cultivar comparisons. The remaining bands can be used freely, even when comparing fresh samples. with samples stored 16 weeks.

The lost cytochrome oxidase bands represented the fastest migrating isoenzymes while the slower isoenzymes maintain both their mobility and density for at least 16 weeks. Two possible explanations for this are the fast migrating isoenzymes are unstable, and the slower migrating isoenzymes are stable; or the faster migrating isoenzymes are subunits which aggregate during storage and form larger complexes which migrate at the same rate as the slower isoenzymes. The slower isoenzymes may in fact be aggregates of subunits, which with proper treatment could be split to yield more sharp individual bands. The proper treatment has not been developed in this study, but may include use of some denaturing agent, chelator, detergent, and/or adjustment of the ionic strength of the extraction solution. Samples to be stained for cytochrome oxidase can be stored for 16 weeks with no loss in density of bands with an R_f less than 0.6. To use all bands in a comparison, fresh material must be used.

Samples stained for phenoloxidase should be compared to samples stored the same length of time, with fresh samples used if bands are to be quantified. As with cytochrome oxidase there are broad, heavy, slow migrating bands present which could possibly be split to yield additional bands.

Except for 1 light band, all polyphenoloxidase bands were present for 16 weeks, suggesting samples to be stained for polyphenoloxidase can be stored for 16 weeks. Again, if band densities are to be quantitatively compared, fresh samples should be used.

According to Wilkinson (33), it is generally better to use fresh material for the study of the relative distribution of the various forms of an enzyme, since certain isoenzymes are frequently less stable than others. Also, some enzymes are stable at room temp and unstable at 4° or −20° while others respond in an opposite manner. The stability of the enzymes included in this study vary, but all may be used after storage within the limitation described.

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