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Preparation of Extracts from Tropical Plants for Electrophoresis — An Analysis of Methodology

Y.Y. GAN
Department of Biology,
Faculty of Science and Environmental Studies
Universiti Pertanian Malaysia,
Serdang, Selangor, Malaysia.

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ABSTRAK

Analisis elektroforesis enzim daripada tumbuhan tropika boleh menjadi sulit kerana kehadiran kadar bahan fenolik yang tinggi dalam tisu tumbuhan. Kadar bahan fenolik yang tinggi dalam tumbuhan tropika itu dipercayai boleh digunakan untuk melindungi tumbuh-tumbuhan daripada pemangsa-pemangsa serangga yang selalu ada di kawasan tropika. Beberapa cara yang berbeza untuk mengekstrak enzim yang perlu sebelum elektroforesis telah dicadangkan. Faktor-faktor yang mengakibatkan protein tumbuhan tak stabil dan susah untuk diekstrak telah juga dikemukakan. Kehadiran tanin dalam tumbuhan diperkenalkan dan tindakbalas-tindakbalas bahan fenolik dengan protein dan enzim telah pun dibincangkan. Teknik-teknik untuk pengekstrakan enzim daripada tumbuh-tumbuhan yang mengandungi banyak tanin mestilah melibatkan penyingkiran substrat fenolik, perencatan aktiviti-aktiviti O-difenoloksidas atau penyingkiran kuinon yang dihasilkan daripada pengoksid dengan menurun kuinon ke O-difenol. Bahan kimia yang dicadangkan untuk ditambah ke dalam medium pengekstrakan terdiri daripada polimer, agen-agen 'copper chelating', 'thiols', askorbat, metabisulfit, ditionit dan bovin serum albumin.

ABSTRACT

Electrophoretic analysis of enzymes from tropical plants can be very difficult due to the presence of high concentrations of phenolics in plant tissues. It is believed that these high tannin levels may serve to protect plants from the numerous insect predators present in the tropics. Different methods of extraction of enzymes prior to electrophoresis are suggested. A number of factors which make plant proteins practically unstable and difficult to work with are also mentioned. The occurrence of tannins in plants and the reactions of these phenolic compounds with proteins and enzymes are discussed. Techniques for the extraction of enzymes from tannin-rich plants should involve the removal of phenolic substrates, the inhibition of O-diphenol-oxidase activity or the removal of quinones formed from oxidation by reducing the quinones back to the O-diphenol. Chemicals added into the extracting medium to overcome the problem of interference by tannins include polymers, copper chelating agents, thiols, ascorbate, metabisulphite, dithionite and bovine serum albumin.

INTRODUCTION

Electrophoresis has been widely used as a useful tool for studies on chemotaxonomy, species phylogenetic relationship, gene pool and other aspects of population and ecological genetics. Electrophoretic protein variations that have been shown to be genetically controlled

through family studies (Lewontin, 1974) are the result of the segregation of alleles at single loci. In cases where family studies are impractical, eg. in long-lived tropical rain forest trees, inferences about the mode of genetic control of the isozymes observed can be made through indirect evidence, such as testing for Hardy-Weinberg equilibrium or by comparisons of the isozymes obtained with those of related species for which family data is available. Such inferences are of course inferior to those that are based on family studies.

Electrophoretic analysis on animals has been more extensively studied as compared to plants (Lewontin, 1974). Most plant electrophoretic studies have used annual angiosperms (Kelly and Adams, 1977; Gottlieb, 1971; 1981; 1982).

The paucity of electrophoretic investigations on perennial angiosperms and gymnosperms is largely due to the presence of endogenous secondary plant products which interfere with the electrophoretic techniques (Kelley and Adams, 1977).

For plant species, research on tropical or subtropical plants is comparatively scarce. The few electrophoretic studies reported include those by Gan *et al.* (1977; 1981 a, b, c; 1982) on some tropical rain forest tree species, on oil palm (*Elaeis guineensis* and *E. oleifera*) and budgrafted mango trees (*Mangifera indica*); by Soltis *et al.* (1980) on ferns (*Bommeria* sp); by Torres *et al.* (1978 a, b, c; 1980 a, b; 1982; 1983) on avocado, date palm and citrus; by Wendel and Parks (1982) on tea; and others on conifers (Kelly and Adams 1977 a, b; 1978; 0' Malley *et al.*, 1979; Mitton *et al.*, 1979; Adams and Joly, 1980 a, b).

We have also attempted to study other tropical plants electrophoretically. These plants include tropical fruit trees such as, durian *(Durio zibethinus),* mangosteen *(Garcinia mangostana),* jack-fruit *(Artocarpus heterophyllus)* and economic plants such as pepper *(Piper nigrum),* bananas *(Musa* cvs.) and sugar cane *(Saccharum officinarum).* When leaves of these plants are ground in buffers, a highly viscous fluid is obtained which browns rapidly. Electrophoresis of this extract yields satisfactory peroxidase and indo-phenoloxidase activities but no activity or smear patterns for all other enzymes.

When electrophoretic analysis first became available (Markert and Moller, 1959), it was thought that crude extracts could be used. In certain organisms this is true but in others, especially in tropical plants, it is not.

Plants produce a large variety and frequently large quantities of secondary products (Robinson, 1975) which cause special problems in the isolation of enzymes, organelles and nucleic acids from their tissues (Loomis 1969; 1974). These secondary plant products interact with proteins in a variety of ways causing inhibition of enzyme activity or otherwise obscuring zymogram patterns (Anderson 1968; Loomis 1969; 1974).

Stahman (1963) and Pirrie (1959) cited a number of factors which cause the extraction of plant enzymes to be difficult. These include tannins, vacuole acids, carbohydrates, hydrolytic enzymes, oxidative enzymes and phytic acids. Dupuis *et al.* (1971) suggested that sesquiterpene lactones with α-methylene groups (found particularly in the Compositae) combine with skin proteins and are allergenic.

Occurrence of Tannins in Plants

The term tannin was introduced by Seguin in 1796. Swain (1965) defined it as being any naturally occurring compound with a high molecular weight (3500,000), containing a sufficiently large number of phenolic hydroxyl or other suitable groups ($1-2$ per 100 MW) to enable it to form cross links with protein and other macromolecules.

It is reported that tropical plants contain high concentrations of tannins. The main role of tannins is to protect plants from the numerous predators present in the tropics (Coley, 1980). Plant tissues which contain tannins are characteristically astringent due to an interaction be-

tween the polyphenols and salivary proteins and glycoproteins in the mouth which cause loss of lubrication. It is thought that this characteristic of tannin reaction prevents damage to the growing plants by browsing animals and predators. Besides being a deterrent to predators, plant tannins also protect plants against diseases caused by fungi, bacteria and viruses (Friend, 1977a). There are often large increases in the synthesis of phenolics in plants after infection by plant pathogens (Farkas and Kiraly, 1962; Friend, 1977b; Kosuge; 1969; Haslam, 1977; Swain, 1979).

Tannins are divided structurally into two groups. The first group is characterised by having a core of a poly-hydric alcohol, such as glucose, the hydroxy groups of which are esterified. Tannins having this structure can readily be hydrolysed by acids, bases or enzymes (tannin hydrolases). They are called hydrolysable tannins (Haslam, 1966).

Tannins of the second group are called condensed tannins. Condensed tannins also from quinones (Swain, 1965) and inhibit enzymes (Loomis and Battaile, 1966).

Both hydrolysable and condensed tannins are widely distributed in plants. In many species both groups are present together, but generally one or the other predominates. Condensed tannins are found in ferns, gymnosperms and angiosperms, but are absent from the nonvascular plants (Swain, 1979).

The Interference by Tannins in the Isolation of Plant Enzymes

In the living plant cell, the protoplasm is protected from the large amounts of potentially damaging phenolic compounds by compartmentalization (Loomis, 1974). When a plant tissue is homogenised in order to isolate enzymes or organelles, this compartmentalization is destroyed and gives rise to undesirable effects.

Endogenous tannins, or quinones and tannins formed after cell rupture inhibit many enzymes extracted from plant tissues (Young, 1965; Loomis 1969, 1974). Thus, isozyme analysis in tannin-rich plants has been restricted to a few enzyme systems encoded by a handful of loci (Gan *et al.*, 1977, 1981; Torres *et al.*, 1978 a, b; O'Malley *et al.*, 1979; Adams and Joly 1980; Wendel, 1980; Watson and Cook, 1981; Wendel and Parks, 1982).

Interactions of Phenols and Quinones with Proteins in Plant Extracts

Animal tissues contain few phenolic compounds (other than tyrosine), and they are seldom in high concentrations (Harborne and Simmonds, 1964), in contrast to plants where the phenolics are widespread and present in high concentrations (Bate-Smith, 1963; Alston and Turner, 1963; Harborne, 1964). Phenols combine with proteins in four ways (Anderson, 1968; Loomis, 1974): (1) by forming hydrogen bonding; (2) by forming ionic bonding; (3) by hydrophobic interactions and (4) irreversibly by oxidation to quinones (Mason, 1959) followed by covalent condensation or by oxidation of protein functional groups by the quinones. The quinones may also oxidize essential groups of proteins. If oxidation of phenols occurs, the resulting quinones may form permament covalent linkages to proteins (Stahmann, 1963).

The oxidation of phenolic compounds is catalysed by phenoloxidases but this only occurs to any appreciable extent when the tissue is homogenised because in intact tissues, the phenolics are spatially separated from the phenoloxidases. Palmer (1963) has reported that phenoloxidases are copper-containing proteins of wide occurrence in nature. They catalyze the aerobic oxidation of certain phenolic substrates to quinones, which are then auto-oxidized to dark brown pigments generally known as melanins. The enzyme phenol-oxidase is of major importance in catalysing the browning of apples (Walker, 1964), bananas (Griffiths, 1959), sweet potatoes (Eiger and Dawson, 1949; Yasunobu, 1959); tea (Yasunobu, 1959) and other fruits and vegetables (Udenfriend *et al.*, 1959).

Therefore, whenever browning occurs in a plant extract or homogenate, it is likely that the

plant proteins are already modified by quinone additions. The proteins may be extensively 'tanned' and converted into dark-coloured insoluble melanoproteins.

Techniques Required for the Extraction of Enzymes in Tropical Plants

The general approach to isolating plant enzymes is to remove phenolic compounds and other secondary products as quickly as possible, and at the same time to prevent the formation of covalent complexes. Therefore, a suitable extraction medium for these tannin-rich plants should:

1. Remove phenolic substrates eg. by absorption.
2. Inhibit O-diphenoloxidase activity, and
3. Remove quinones formed from oxidation by reducing the quinones back to the O-diphenol.

The following chemicals have been suggested for addition to the extraction medium (Anderson, 1968; Loomis, 1974):

a. *Polymers.* Soluble PVP (Polyvinyl-pyrrolidone) was used by Hulme *et al.* (1964) for the preparation of mitochondria from apple peel free from the contamination of phenols. Harel *et al.* (1964) found that PVP inhibits purified preparation of O-diphenol oxidase from apple fruits.

PVP was found to be effective in the extraction of enzymes from tobacco leaf (Pierpoint, 1966), though the mechanism of this inhibition is uncertain. Loomis and Battaile (1966) thoroughly reviewed the use of insoluble PVP for the removal of plant phenols and quinones from extracts. Using this compound as an additive, they successfully extracted peppermint, thistle and apple leaf proteins. An insoluble form, known as polyclar AT, is very effective in isolating plant enzymes, since it binds phenols as an insoluble complex and allows rapid separation of phenols from soluble proteins. The pH of the extraction medium should be low enough to suppress ionization of phenolics, a pH of 7.2 or lower is probably preferable (Jones *et al.*, 1965; Sanderson, 1966). Anderson and Rowan (1967) suggested that the function of PVP is to inhibit O-diphenoloxidase, to bind phenolics and tannins, and thereby remove endogenous substrates of O-diphenoloxidase. Other polymers have also been successfully used. Examples are polyethylene glycol (Bandran and Jones, 1965) and polycaprolaktam (Young, 1965).

b. *Copper chelating agents.* DIECA (diethyldithiocarbamate) is a most efficient inhibitor of O-diphenoloxidase (Bonner, 1957; Walker, 1964; Pierpoint, 1966), because it chelates with copper which forms the active centre of this enzyme. Furthermore, DIECA combines with the quinone product.

DIECA has been successfully used for the extraction of sucrose synthetase from sugar cane (Slack, 1966).

Cyanide is also a powerful inhibitor of O-diphenoloxidase (Anderson and Rowan, 1967). Palmer and Roberts (1967) reported that very low concentrations of mercaptobenzo-thiazole inhibit O-diphenoloxidase and that the inhibition is reversed by equally low concentrations of cupric ions. They suggested that mercaptobenzothiazole combines with copper at the active centre of O-diphenoloxidase, thus making an inactive complex. Ethyl xanthate (Harrison and Pierpoint, 1963; Pierpoint and Harrison, 1963) was also found to be useful in the extraction of plant enzymes.

c. *Thiols.* Activity of enzymes and mitochondria in extracts prepared from plant tissues is greatly enhanced by including thiols in the extracting medium. These thiols include cysteine, thioglycollate, glutathione and mercaptoethanol (Hageman and Waygood, 1959; Kleinhofs *et al.*, 1967).

Thiols can be arbitrarily grouped into two classes, depending on the type of reaction between thiol and quinone. One class reduces quinones back to the O-diphenol and

includes thiols such as mercaptobenzothiazole (Pierpoint, 1966; Palmer, 1965; Palmer and Roberts, 1967) and thioglycollate (Pierpoint, 1966) which tend to be powerful inhibitors of diphenoloxidase. The other class combines with quinones to form a product which is not further oxidized and does not inhibit enzymes; examples of these thiols are cysteine (Henze, 1956; Walker, 1964), DIECA and ethyl xanthate (Pierpoint, 1966). This latter class tends to be less powerful in inhibiting O-diphenol oxidase than the first class.

Glutathione and thiourea (Henze, 1956) also combine with quinones.

d. *Ascorbate.* Ascorbate readily reduces quinones with the regeneration of the phenol and is widely used for this reason to measure O-diphenoloxidase activitiy (El-Bayoumi and Frieden, 1957; Palmer, 1965).

e. *Metabisulphite and Dithionite.* Anderson and Rowan (1967) reported that metabisulphite could inhibit O-diphenol oxidase completely. It is more efficient than thiols in preventing inactivation of tobacco leaf peptidase during extraction. Such inactivation is permanent and the minimum concentration of metabisulphite required to provide maximum protection does not vary with the physiological state of the tissue nor with the amount of the tissue extracted per volume of extracting medium. Palmer (1965) has suggested that dithionite is rapidly oxidized, thus depleting the assay mixture of oxygen and inhibiting O-diphenoloxidase.

f. *Bovine Serum Albumin (BSA).* BSA is routinely included for extracting plant mitochondria (Verleur, 1965; Stokes *et al.*, 1968; Ikuma and Bonner, 1967), because it absorbs by an uncoupled plant mitochondria (Dalgarno and Biat, 1963). Weinbach and Garbus (1966a, b) demonstrated that BSA powerfully binds phenolics and reverses the uncoupling effects of phenols of low molecular weights. On the other hand, mitochondria from potato tuber, extracted and assayed with BSA in the medium, are uncoupled unless either cysteine (Verleur, 1965) or metabisulphite are included in the extracting medium.

MATERIALS AND METHODS

When all enzyme systems (except peroxidase and indole-phenoloxidase) are 'lost' and cannot be stained after electrophoresis, it is necessary to carry out tests to determine: (a) whether the extract was too dilute, (b) whether the staining methods were appropriate, (c) whether the electrode buffers and/or gel buffers were suitable, (d) whether the pH for electrophoresis was optimal and (e) whether it was related to the different concentrations of the gels used.

The amount of leaf tissue used for extraction should also be reduced to check whether it is due to overloading. Meanwhile, effects of storage should be tested since they might cause deterioration of proteins. However none of these tests give positive or encouraging results. So finally, chemicals which are naturally present in plants and which are known to cause inhibition of enzyme activity during extraction should be tested.

The majority of these chemicals suggested in the literature were tried individually and in different combinations in the extracting medium for tannin-rich tropical plants. They included: (1) Mercaptoethanol, (2) PVP-10 and PVP-40, (3)DIECA, (4) Charcoal (absorbs the brown colour of the plant extract), (5) Cysteine, (6) Metabisulphite, (7) Urea, (8) Polyclar AT, (9) Thioglycollate, (10) Glutathione, (11) Ascorbate and (12) BSA.

They were tested at different concentrations (5 – 100 mM) and different pH values (7 – 8.6). When these chemicals were used individually, there was some indication of band differentiation but resolution was still far from satisfactory. These chemicals were then tried in all possible combinations of two or three compounds. According to the nature of their reactions mentioned above, they were divided into the groups

as shown in Table 1. Any two or three chemicals belonging to different groups were tried in combination systematically. For example, the combinations were as follows:

1. PVP-10 or PVP-40 + DIECA
2. PVP-10 or PVP-40 + Cysteine
3. PVP-10 or PVP-40 + Thioglycollate
4. PVP-10 or PVP-40 + Glutathione etc.,

and the same series was repeated for polyclar AT in group 1. Then the same procedure was worked out between group 2 DIECA and the remaining chemicals in others groups, and so on.

Electrophoresis was conducted at 4°C for 4 hours on starch gels. 40 gram of starch (Connaught hydrolysed starch) was dissolved in 300 ml of gel buffers.

TABLE 1
Effective chemicals used for electrophresis of tannin-rich plants grouped according to their chemical properties

Group No.	Type	Chemicals
1	Polymer	PVP-10
		PVP-40
		Polyclar-AT
2	Copper chelating agent	DIECA
3	Thiols	Cysteine
		Thioglycollate
		Glutathione
		Mercaptoethanol
4		Charcoal
5		Arcorbate
6		Metabisulphite
7		Urea
8		BSA

After electrophoresis, gels were cut into half horizontally and each gel was stained for two enzyme systems. The staining recipes are as follows: *Esterase,* 1 ml of α-naphthyl acetate (0.186 gm/10 ml acetone), 1 ml of β-napthyl acetate (0.186 g/10 ml acetone), 0.1 gm fast blue B, 30 ml of Na_2HPO_4 (21.33 gm/l) and 70 ml of NaH_2PO_4 (20.44 gm/1). *Catalase,* 100 ml of 0.5% KI acidified with 0.5 ml of glacial acetic acid and 100 ml of 0.5% H_2O_2. *Leucine aminopeptidase,* 50 ml Tris A (19.61 gm maleic anhydride and 14.22 gm of Trizma base in 1 litre of distilled water, pH 3.6), 10 ml Tris B (6 g NaOH in 1 liter of distilled water, pH 12.2), 50 mg fast garnet GBC, 40 mg l-leucyl-naphthylamide-HCl dissolved in 1 ml of acetone, 40 ml distilled water. *Acid phosphatase,* 100 mg α-naphthyl acid phosphate, 250 mg PVP, 1 gm NaCl, 10 drops 10% $MgCl_2$, 100 mg fast garnet GBC and 100 ml 0.05 M acetate buffer, pH 5.0. *Alkaline phosphatase,* 100 mg Na- β-naphthyl phosphate, 100 mg fast blue RR, 123 mg $MgSO_4.7H_2O$ and 100 ml 0.06 M borate buffer, pH 9.7. For the enzymes esterases, catalase, leucine aminopeptidase the gel buffer was 0.065 M Trizma base, 0.01 M citric acid, pH 8.7, the box buffer was 0.2 M boric acid, 0.025 M lithium hydroxide, pH 8.5. For the acid and alkaline phosphatases, the gel buffer was 9.2 gm Trizma base, 1.05 gm citric acid in 1 litre of distilled water, pH 8.6, the box buffer was 18.55 gm boric acid, 50 ml of 1M NaOH to 1 litre of distilled water, pH 8.0.

RESULTS AND DISCUSSIONS

Tropical Rainforest Species

The results from these different combinations which gave improved patterns for the extraction of *Xerospermum intermedium* (Sapindaceae), a wide relative of the rambutan are presented in Table 2. The number of "+"s reprents the degree of improvement.

Loomis and Battaile (1966) reported that soluble PVP is a very effective agent for removing phenolics in the isolation of plant mitochondria, and that insoluble PVP (polyclar-AT) is effective in isolating soluble plant enzymes.

TABLE 2

Zymograms of *Xerospermum intermedium* were improved after the addition of chemicals in extraction as listed below. The number of +s represents degree of improvement

1.	PVP + DIECA + Cysteine	+ +
2.	PVP + DIECA + Thioglycollate	+ +
3.	PVP + DIECA + Glutathione	+ +
4.	PVP + DIECA + Mercaptoethanol	+ + + +
5.	Polyclar-AT + DIECA + Cysteine	+ +
6.	Polyclar-AT + DIECA + Thioglycollate	+ +
7.	Polyclar-AT + DIECA + Glutathione	+ +
8.	Polyclar-AT + DIECA + Mercaptoethanol	+ + +
9.	DIECA + Charcoal + Mercaptoethanol	+ +
10.	Charcoal	+ (colourless extract)
11.	Metabisulphite + PVP + Mercaptoethanol	+ + +
12.	Metabisulphite + PVP	+ +
13.	Metabisulphite + Mercaptoethanol	+ +
14.	Urea + PVP	+
15.	Metabisulphite + DIECA	+ +
16.	Ascorbate + PVP	+ +
17.	Ascorbate + PVP + Mercaptoethanol	+ +
18.	Ascorbate + DIECA + PVP	+ + +
19.	Ascorbate + DIECA + Mercaptoethanol	+ +
20.	Ascorbate + Metabisulphite + DIECA	+ + +
21.	Ascorbate + PVP + Metabisulphite	+ + +
22.	BSA + Ascorbate + PVP	+ +
23.	BSA + DIECA + Ascorbate	+ +
24.	BSA + DIECA + PVP	+ + +
25.	Metabisulphite + Polyclar-AT	+ +
26.	Metabisulphite + Polyclar-AT + Mercaptoethanol	+ +
27.	Ascorbate + Polyclar-AT	+
28.	Polyclar-AT + Mercaptoethanol	+ +
29.	Urea + Polyclar-AT	+
30.	BSA + Polyclar-AT	+ +

In the present work, soluble PVP gave better results than polyclar-AT. Since PVP is soluble is water, it is very easy to handle, the viscous extraction buffer containing soluble PVP is used directly to macerate plant tissue and it is immediately soaked with filter paper and then slotted in the gel directly without centrifugation. In this way, the best extraction medium for *X. intermedium* was obtained by adding 10mM PVP + 10mM DIECA + 0.2% mercaptoethanol at pH 8.6 0.2 M Tris-HCl.

Oil Palm (Elaeis guineensis)

The extraction of enzymes from the leaves of oil palm also gave similar problems. When the usual extraction buffer was used, no clear bands could be identified in the zymogram. All the enzymes (except peroxidase and indole-phenol-oxidase) gave smearing effect i.e. the middle portion of the gel stained uniformly dark without differentiating into bands. After the addition of the reducing agents suggested in Table 1, improved patterns were obtained. The results of the different combinations of chemicals added in the extraction buffer are presented in Table 3. Similarly, the number of +s represents degree of improvement. The combination of ascorbate, PVP, metabisulphite and DIECA gave the best zymogram patterns and therefore the modified extraction buffer for oil palm is suggested as follows: 1 ml of standard extraction buffer (0.2

TABLE 3

Zymograms of oil palm (*E. guineensis*) were improved after the addition of the chemicals in extraction as listed below. The number of + s represents degree of improvement

1.	PVP + DIECA + Mercaptoethanol	+ +
2.	Polyclar-AT + DIECA + Mercaptoethanol	+ +
3.	Metabisulphite + PVP + Mercaptoethanol	+ +
4.	Ascorbate + metabisulphite + DIECA	+ + +
5.	Ascorbate + PVP + Metabisulphite + DIECA	+ + + +
6.	PVP + DIECA + Glutathione	+ +
7.	Metabisulphite + PVP	+ + +
8.	Polyclar-AT + DIECA	+ +
9.	PVP + DIECA + Thioglycollate	+ +
10.	Polyclar-AT + DIECA + Cysteine	+ +

M TRIS-HCL with 10% sucrose, 1% mercaptoethanol, pH 8.6) is mixed with 0.5 ml 100 mM PVP-10, 0.25 ml 10 mM ascorbate, 0.25 ml 10 mM metabisulphite and 0.5 ml 10 mM DIECA.

By using this extraction buffer, clear and repeatable zymograms can be obtained.

For other plant species, inhibition of enzymes by phenolic compounds has also been reported in peppermint (Loomis and Battaile, 1966). The extraction of mitochondria is inhibited by the reaction of phenoloxidase in sweet potatoes (Lieberman and Biale, 1965) and apples (Hulme *et al.*, 1964). When apple mitochondria were extracted with the addition of PVP, no inhibition of mitochondria was observed (Hulme and Jones, 1963; Harel *et al.*, 1984; Walker and Hulme, 1965). Hoover *et al.*, (1977) reported that chlorogenic acid, at 0.4 mM, inhibited G6PDH completely. The degree of inhibition decreased when the concentration of chlorogenic acid was reduced.

Oxidation of phenols may be catalyzed by either phenoloxidase or peroxidases, or it may occur nonenzymatically. This is the reason why only peroxidases and indole-phenoloxidase can be obtained in the electrophoresis of the leaves of oil palm when the ordinary extraction buffer is used. All other enzymes were completely lost no matter what buffer system was used during electrophoresis. This is because most enzymes are precipitated during the conventional extraction procedure. Some phenols may inactivate or modify enzymes without precipitating them (Loomis, 1974).

It is possible to overcome the problems caused by the presence of tannis and obtain good zymograms. Nevertheless, due to the great chemical diversity in plant tissues, no general formula for an enzyme extraction buffer suitable for all plant species can be given. Probably every tannin-rich plant species needs a different extraction buffer as each plant species may require different amounts of adsorbents and the most effective combinations of these may also vary. This paper therefore aims to provide guidelines in methodology for further studies in the electrophoretic analysis of tannin-rich tropical plants.

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