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Review

Application to plant proteins of gel electrophoretic methods

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Abstract

The present review describes the major extraction and gel electrophoretic procedures available for the study of plant proteins. Firstly, some technical considerations are made about the extraction of plant proteins, which necessitates special precautions due to the presence in plant cells of several substances that may readily and severely alter the integrity of the proteins extracted. After a brief discussion on the utility of gel electrophoresis for the study of plant enzymes involved in plant growth and development, some of the most useful techniques available to study plant defense proteins are then described. Techniques allowing the specific detection of hydrolases acting on microbial cell walls, in particular, are described in detail. The detection on gels of some inhibitors involved in the resistance of plants to insect pests is also considered.

Contents

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1. Introduction

Gel electrophoresis of proteins constitutes a powerful and useful tool for the study of plant growth and development. Characterized by an extremely complex metabolism, plants produce a multitude of proteins involved in various cellular, physiological and defense processes [1,2]. While current gel electrophoretic methods such as sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), native PAGE, two-dimensional (2D) PAGE and immunoblotting have been widely used to study plant proteins, more specific techniques have also been reported. In particular, activity gel strategies have been adapted for the detection and assay of several plant enzymes, especially those involved in defense mechanisms [3]. In parallel, other techniques have been proposed to analyze in gels or after blotting onto solid matrices post-translational modifications of plant proteins, especially those associated with glycosylation processing [4,5].

After discussion of the major points to consider for an adequate extraction of plant proteins, this review describes some of the most widely used techniques developed for plant enzyme and inhibitor characterization. Due to space constraints, the use of gel electrophoresis for comparative analyses of protein patterns, which represents an important field of plant protein analysis, is not covered in this review. Also, the powerful techniques devised to study plant protein glycosylation are not discussed, since an extensive review on this topic has been presented earlier [6].

2. Extraction of plant proteins

Protein extraction is often considered as a simple preliminary step for subsequent purification or electrophoretic procedures but in fact constitutes a crucial step when studying plant proteins. The extraction procedure determines both the nature and the stability of the proteins extracted, which in turn determine the quality and the validity of subsequent results obtained by electrophoretic analyses. In particular, the extraction of plant proteins raises specific problems inherent to cell content. In comparison with animal tissues or mibrobial cells, plant tissues have a much lower protein content and the vacuole, which occupies a large part of the cell, contains numerous compounds which have a deleterious effect during protein extraction. Importantly, the choice of a specific extraction strategy must be based on the data available about the spatial localization of proteins under study and on the sample tissue characteristics, especially those potentially interfering with the integrity of the extracted proteins.

2.1. Choosing an extraction procedure

The diversity of proteins involved in plant growth, development and defense processes renders unlikely the formulation of a universal

extraction procedure to recover all proteins of a given tissue in a form suitable for subsequent electrophoretic analyses. However, the solubility of plant proteins, which is closely associated with their intracellular localization, helps to formulate three general approaches: (1) aqueous buffer extraction, (2) detergent extraction and (3) direct precipitation. These general extraction approaches may be further adapted for each specific case.

2.1.1. Aqueous buffer extraction

The first strategy consists of extracting soluble proteins in a non-denaturing aqueous buffer [e.g. Tris, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES), phosphate] with either a low or high ionic strength (IS). While the use of a low-IS buffer allows the recovery of soluble proteins from both the cytoplasm and the cellular compartments [7,8], the use of a high-IS buffer also allows the extraction of cell wall-bound proteins [9,10]. Particularly useful for subsequent activity studies using electrophoretic gels, this non-denaturing approach often necessitates some precautions to ensure the stability of the extracted proteins (see Sections 2.2 and 2.3).

2.1.2. Detergent extraction

The second strategy is based on the use of the detergent SDS and allows the extraction of both soluble and membrane-bound proteins under denaturing conditions [11,12]. After extraction, SDS may easily be removed by ultrafiltration or dialysis, thereby allowing the renaturation of the proteins and subsequent activity studies on gels. The efficient solubilization of membrane proteins in the presence of SDS, however, necessitates an elevated temperature which may cause the irreversible precipitation or denaturation of certain proteins.

2.1.3. Direct precipitation

The third extraction strategy consists of directly precipitating total protein using cold 10% (w/v) trichloroacetic acid (TCA) [12,13]. This approach is useful either when nothing is known about the cellular emplacement of proteins under study or when simply comparing protein patterns from different extracts (e.g. 2D-PAGE systems). The TCA-precipitation procedure does not allow subsequent activity studies on gels, since TCA irreversibly inactivates enzymes, which cannot resume their catalytic functions when resuspended in a buffer [13]. Where activity has not to be maintained, however, this extraction strategy may prove particularly useful when used in combination with aqueous buffer and SDS extractions to fractionate proteins according to their localization in cells (Table 1).

Apart of the three strategies described, several other extraction procedures such as acetone precipitation [14] and TCA–acetone precipitation [15] have been described. The choice of a given strategy finally depends on the specific characteristics of the proteins. The extraction of some proteins localized at the interface between the cytoplasm and the cell membrane, for instance, necessitates a very-low-IS buffer to ensure their recovery from the cell wall fragments after tissue breakage [16]. The extraction of amphipathic proteins, which contain both water- and membrane-soluble domains or subunits, may also necessitate particular extraction conditions. Extracellular proteins, finally, may be specifically extracted with intercellular fluids by washings in low-IS buffers. Such an extraction, which requires in vacuo infiltration of the tissue in water (or in low-IS buffers) followed by low-speed centrifugation recovery of extracellular washings, notably allows the selective extraction of several plant defense proteins [3,17].

Whatever the procedure chosen, however, care must be taken to ensure the stability of proteins after breakage of the plant tissue. Mature plant cells, especially those from green tissues, present special problems in the isolation of proteins, primarily owing to the presence of proteases and phenolic compounds in their vacuoles. During the extraction procedure, these substances are released from broken cells and may severely alter the proteins. Phenolic compounds, which are found in large amounts in some tissues, may also interfere with the estimation of protein levels. When studying plant proteins, these interfering processes must be taken into account, since they could influence

Table 1
General strategy for the fractionation of plant proteins during extraction

Desired proteins	Extraction procedure		Fractionation
	Step 1[a]	Step 2[b]	
Soluble (s)	Aqueous buffer (low IS)	–	Step 1: s
Membrane (m)	Aqueous buffer (low IS)	SDS extraction	Step 1: s Step 2: m
Cell wall (cw)	Aqueous buffer (low IS)	Aqueous buffer (high IS)	Step 1: s Step 2: cw
s + m	SDS extraction	–	Step 1: s + m
s + cw	Aqueous buffer (high IS)	–	Step 1: s + cw
m + cw	Aqueous buffer (low IS)	TCA extraction	Step 1: s Step 2: m + cw
s + m + cw (total)	TCA extraction[c]	–	Step 1: s + m + cw

[a] Extraction from fresh tissue.
[b] Extraction from the resulting pellet.
[c] Some integral membrane proteins may not be extracted at low temperature [12]. A subsequent extraction with SDS at high temperature may allow their recovery from the pellet.

results subsequently obtained by electrophoretic analyses.

2.2. Avoiding protein degradation

Protein degradation is a common phenomenon observed during the extraction of proteins from either microbial, animal or plant sources [18]. In protease-rich tissues, degradation can occur as soon as the cells are broken, leading to a significant alteration of protein patterns observed in gels [12,13]. Proteolytic activity in the developing anther of petunia, for example, is so severe that high-molecular-mass proteins are degraded within a few minutes after homogenizing tissues in buffered solutions [13]. Similarly, the M_r 220 000 structural protein β-spectrin from tomato leaf is not detected after SDS-PAGE when proteolytic activity in the sample is not blocked by inhibitors of proteolysis [16].

A simple way to prevent plant protein degradation during extraction consists of inactivating proteases by grinding the tissues in a buffer containing SDS [11,12]. Such an approach, however, does not always give satisfactory results, since some proteases remain active in the presence of the detergent [13,19]. Denaturation by SDS may even, in some cases, accelerate proteolysis by exposing sites on target proteins without inactivating the proteinases [18]. In addition, some proteinases inactivated by SDS can survive fairly harsh denaturation conditions (e.g. heating samples with SDS and β-mercaptoethanol) and recover their activity after removal of denaturing agents [18].

Alternatively to SDS extraction, two strategies may be considered: (1) extraction of the tissues in cold TCA and (2) addition of protease inhibitors (PIs) to the extraction buffer. The first approach, which consists of grinding the tissues in 10% (w/v) cold TCA, provides a fast method for analyzing proteins in their undegraded form [13]. The use of TCA is particularly useful in preventing misinterpretation of protein patterns in SDS–polyacrylamide gels resulting from partial hydrolysis of proteins during sample processing. As noted above, however, activity studies on gels may not be performed after TCA precipitation. When recovery of activity is required, the use of PIs appears more appropriate. Simply, this approach consists of adding to the extraction buffer a "cocktail" of PIs composed of inhibitors with affinity for the four major mechanistic classes of proteases (Table 2). While some PIs cause irreversible inhibition [e.g. phenylmethylsulfonyl fluoride (PMSF), *trans*-epoxysuc-

Table 2
Protease inhibitor cocktail

Inhibitor	Target proteinases	Effective concentration	Stock solution
E-64	Cysteine	10 μM	1 mM in water
EDTA	Metallo-	2 mM	500 mM in water
Leupeptin	Serine, (cysteine)	10 μM	10 mM in water
Pepstatin	Aspartate	1 μM	1 mM in methanol
PMSF	Serine, (cysteine)	1 mM	100 mM in methanol

cinyl-L-(4-guanidino)butane (E-64)] and can easily be removed without regeneration of protease activity, others are reversible inhibitors (e.g. pepstatin, leupeptin) that cannot be removed or diluted without risks of protease renaturation. Although the use of PIs has proven useful to decrease proteolytic activity in a variety of tissue extracts, it is important to keep in mind that the inhibitors must be carefully chosen regarding the extraction or purification procedure to be used.

The nature of the tissue has also to be considered. Plant tissues often contain serine and cysteine proteases which can be efficiently inhibited by PMSF in combination with leupeptin or E-64, but PIs have not proven useful for all systems. Some plant proteases were shown to retain their activity in urea even in the presence of PIs [20,21]. In such cases, specific extraction conditions such as a high pH [22] or a low temperature may provide adequate protection of proteins. Table 3 summarizes some experimental conditions useful to avoid proteolysis during extraction.

2.3. Avoiding protein–phenolics interactions

Many plant tissues are known to contain large amounts of phenolic compounds which, either in their primary form or oxidized to quinones, combine irreversibly with proteins [23]. Phenols bind to proteins by forming strong hydrogen bonds with the oxygen atoms of peptide bonds or, when oxidized to quinones, by condensing with –SH and $-NH_2$ groups of proteins. These chemical interactions lead to the formation of protein dimers and polymers cross-linked by polyphenols [24], thereby affecting the quality of protein patterns observed in gels.

An efficient way to avoid alteration of proteins

Table 3
General rules to avoid protein degradation during extraction

Temperature and time	Activity of proteases is decreased by working at 4°C. Alternatively, samples should be placed on ice throughout the extraction process. The extraction period should also be minimized.
pH	pH buffers either above or below optimal pH for proteolytic activity may help to prevent proteolysis. The choice of an appropriate pH must be considered for each specific extract according to the endogenous proteases.
Stabilizing agents	Some reagents such as dimethyl sulfoxide (10%, v/v) or glycerol (25%, v/v) may protect proteins against proteases. The addition of reducing agents such as dithiothreitol (1 mM), L-cysteine (5 mM) or β-mercaptoethanol (1 mM) may protect proteins from oxidation, but care must be taken in the presence of cysteine proteinases that are activated in reducing conditions.
Exogenous proteins	Where purity of the proteins is not essential, the addition of exogenous proteins such as bovine serum albumin (1 mg/ml) may stabilize proteins by providing an alternative substrate for proteases.
Chelating agents	Chelators such as EDTA (2 mM) or ethyleneglycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA, 2 mM) should be added to the extraction buffer for removing divalent cations, which are cofactors of metalloproteinases and several serine proteases. Alternatively, divalent cations may be excluded from the extraction buffer.

by phenolic compounds simply consists of precipitating all proteins by grinding the tissues in cold TCA [13]. When biological activity has to be maintained, the effects of phenolics and quinones may be avoided by the addition of polyvinylpyrrolidone (PVP), which binds to phenols [7,25–28]. In combination with PVP, the inactivation of phenol oxidases by the addition of reducing agents such as ascorbate [25,27–29], β-mercaptoethanol [30–34], potassium hydrogensulfite [35], dithiothreitol (DTT) [7,27] or thiourea [26,36] may be useful. By using a low-pH extraction buffer, which helps to prevent formation of non-enzymatically generated quinones [26] and favors the binding of PVP to phenols [37], the use of these compounds adequately protects plant proteins extracted from various tissues. The use of antioxidant substances, however, must be considered with care, since some tissues contain cysteine proteinases that are strongly activated under reduced conditions [38,39]. A more appropriate approach would be, in this case, the use of detergents. SDS, for example, is particularly useful for inactivation of phenol oxidase activity in pine needle extracts, a material rich in phenols and terpenes [40]. Alternatively, the removal of phenolic compounds by adding Triton X-114 to the extraction buffer can be worthwhile. Useful for the purification of integral membrane proteins [41], this detergent efficiently sequesters plant phenolics, leaving phenolic-free proteins in the aqueous phase [42,43]. The simultaneous use of Triton X-114 and PIs could allow an adequate protection of plant proteins isolated from phenolic/protease-rich tissues.

2.4. Assaying protein levels in plant extracts

Estimation of total protein in plant extracts is, as for animal and microbial extracts, an important procedure for an adequate interpretation of protein patterns in electrophoretic gels. Although the general methods of Lowry et al. [44] and Bradford [45] are widely used to estimate protein contents in various samples, they are primarily adapted to assay proteins of animal or microbial origin. Given the presence of phenolic compounds in plant cells, the procedure used for assaying plant proteins must be carefully chosen. The Lowry et al. [44] and biuret methods, which are based on the quantification of phenolic compounds, are not suitable to properly quantify plant proteins, especially those from green tissue extracts. Due to less interference from phenolics, the Bradford [45] assay appears more appropriate. When used in conjunction with extraction procedures minimizing protein–phenolics interactions (PVP, reducing agents, TCA), it can give protein estimations similar to those obtained with the Kjeldahl method (see [46]). For electrophoretic studies, where relatively rough estimations of protein contents are in several cases appropriate, the Bradford assay [45] is the method of choice for rapid and simple assay of plant proteins.

It is important to note, however, that the Bradford assay is based on the action of Coomassie Brilliant Blue G-250, a dye which does not react equally with all proteins [45,47,48], and that it cannot be used for fine comparisons of protein contents extracted from different tissues. Alternatively, Marks et al. [49] proposed an acid protein hydrolysis in 3% (v/v) sulfuric acid followed by a quantification of amino acids by the ninhydrin method. Despite the loss of some amino acids during acid hydrolysis [50], the release of total amino acids is found to be linearly related to the original amounts of protein present. Also, the quantification of total protein by ninhydrin, which appears to be relatively independent of the nature of the amino acids present [49], is not influenced by phenolic compounds. Where precise estimations are desired, this method may prove useful for various plant protein extracts. The estimation of protein content by the Kjeldahl procedure, which utilizes the linear relationship between nitrogen and protein contents (factor 6.25), is also frequently used to estimate protein content from plant extracts. A major limitation of this method, however, is that it does not allow discrimination between protein nitrogen and the other forms of the element (inorganic N, alkaloids, etc.). The conversion factor of 6.25 is also inexact in some cases (e.g. for tropical plant

species [51]) and may lead to erroneous estimation of total protein.

3. Gel electrophoresis of plant proteins

Gel electrophoretic assays have been proposed for various enzymes and inhibitors involved in either microbial, animal and/or plant metabolism. Interestingly, and given the relative conservation of enzymatic functions during evolution, techniques developed for microbial or animal systems may often be easily adapted for plants, extending the spectrum of techniques available for plant enzyme analysis. The remarkable diversity of plant defense proteins intended to limit growth of pathogens and pests has also led to the development of several procedures primarily adapted to plant systems.

3.1. Studying growth and development

Enzymes involved in either microbial, animal or plant metabolism may be classified as oxidoreductases, transferases, hydrolases, lyases, isomerases, ligases or nucleic acid-modifying enzymes depending on their respective mode of action and substrate specificities. Despite the crucial importance of these enzymes in plant metabolism, the numerous gel electrophoretic procedures developed or adapted for their analysis are not described in this review, since two extensive and excellent reviews were recently published on this topic [52,53]. Some studies recently published on the specific topic of gel electrophoresis of plant metabolic enzymes are listed in Table 4.

Table 4
Assay of plant enzymes by gel electrophoresis: list of recent studies

Enzyme class	Enzyme	Ref.
Hydrolases	Acid phosphatase	[54]
	Esterase	[55,56]
	Phosphatases	[54,57]
	Proteases (*endo*)	[58]
	Proteases (*exo*)	[59]
Isomerases	Phosphoglucose isomerase	[56]
Oxidoreductases	Malate dehydrogenase	[56]
	Oxidases (ascorbate)	[60]
	Peroxidases	[61,62]
	Peroxidases (ascorbate)	[63]
	Superoxide dismutase	[56,64]
Transferases	Glycosyltransferase	[65]

3.2. Studying defense responses/resistance to pathogens

Certain plant proteins possess the capacity to act as potential antimicrobial agents by altering the integrity of cell walls required to preserve the shape and internal pressure of microbial cells [3]. In recent years, several gel electrophoretic procedures have been described to study these important proteins. These procedures are based on the biological activity of the defense proteins, which usually display lytic activity against bacterial or fungal cell wall components.

3.2.1. Enzymes acting on bacterial walls

Plants produce some enzymes that can degrade the cell wall components of bacteria. In several cases, hydrolysis of bacterial walls is brought about by enzymes active on peptidoglycan, a β-1,4 polymer of alternating N-acetyl-D-glucosamine and N-acetyl-D-muramic acid residues linked to various peptide and carbohydrate components [66]. Some peptidoglycan hydrolases have been characterized as either N-acetylmuramidases (lysozymes), N-acetylglucosaminidases, N-acetyl-L-alanine amidases, endopeptidases or transglycosylases depending on their substrate specificity [67]. Produced by plants as a defense mechanism, it is also noteworthy that bacterial cells influence their own growth by producing autolysins, which catalyze the lysis of their own cell walls [68].

A simple PAGE assay for detection of bacterial cell wall hydrolases consists of incorporating cell walls of Gram-positive bacteria at a final concentration of 0.2% (w/v) into polyacrylamide slabs gels. Bacterial extracts (endogenous autolysins) or plant extracts (exogenous bacterial cell wall hydrolases) are subjected to SDS-PAGE

in gels containing the entrapped substrate. After electrophoresis, renaturation of enzymes is performed in the presence of purified and buffered 1% (v/v) Triton X-100 to efficiently remove SDS from denatured proteins. After incubation in appropriate buffers, cell wall hydrolases are visualized as clear lytic areas against the opaque cell wall substrate background. Lytic zones in gels can be seen as clear or opalescent bands within the opaque greyish substrate when examined with light from the back. Lytic zones can be photographed by transparency against a dark background and thus visualized as dark bands on photographs (Fig. 1 and Ref. [69]). One to fifteen bands with lytic activity may easily be detected, depending on bacterial extracts and on the nature of the cell walls incorporated as substrate into gels [69]. This simple PAGE assay

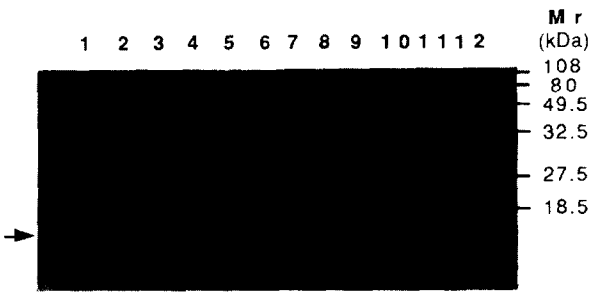

Fig. 1. Electrophoretogram of lysozyme activity assay with transgenic potato expressing hen egg white lysozyme (HEWL). Samples of 5 μl (20 μg protein) of leaf homogenates (1:3, w/v, in denaturing sample buffer [72]) from 12 HEWL-transgenic potato plantlets (1–12) were separated in a 15% (w/v) polyacrylamide denaturing (SDS) slab gel containing 0.2% (w/v) *Micrococcus luteus* lyophilized cells as lysozyme substrate [72]. After electrophoresis, the separating gel was incubated for 1 h in 50 m*M* sodium phosphate buffer (pH 7.0) containing 1% (v/v) purified Triton X-100 at 37°C with gentle agitation [72]. The lysis bands (lysozyme activities) appeared as dark bands on the greyish opaque substrate when the gel was observed against a black background. All samples exhibited lysozyme activity, exept for potato samples 2 and 9. Numbers on the right (M_r) refer to molecular mass markers (kDa = kilodalton). The arrow on the left corresponds to HEWL activity at M_r 14 400. Note that the same type of electrophoretograms and photographs are obtained (dark bands against an opaque background) when insoluble enzyme substrates are incorporated into the separating gel and hydrolyzed by enzymatic activities. Gel assay made by J. Trudel.

of bacteriolysis was successfully used to screen and characterize a bacteriolytic enzyme from *Bacillus* [70] and a *Streptococcus* autolysin [71].

The best characterized plant enzymes acting on bacterial walls are lysozymes (EC 3.2.1.17; muramidases or peptidoglycan N-acetylmuramoyl-hydrolases), which are endohydrolases cleaving the peptidoglycan (murein) between the C-1 of N-acetyl-D-muramic acid and the C-4 of N-acetyl-D-glucosamine [66]. As lysozyme activity is easily assayed by lysis of *Micrococcus luteus* (syn: *lysodeikticus*) cells as substrate, a simple PAGE assay of plant lysozymes consists of incorporating a *M. luteus* cell suspension substrate into polyacrylamide slab gels. *M. luteus* can be directly incorporated into SDS gels when proteins are separated by SDS-PAGE [72] or incorporated into a separate overlay gel when proteins are subjected to native PAGE [73]. As previously described for bacterial walls [69], lysozyme activity is revealed and visualized as clear or opalescent lysis bands against the greyish opaque background of 0.2% (w/v) autoclaved *M. luteus* cell suspension [72,73]. This lysozyme assay after PAGE was recently used to determine the fate and activity of a cloned hen egg white lysozyme in transgenic tobacco [74] and potato (Fig. 1) plants. It was also used to characterize further some plant lysozymes [75,76].

3.2.2. Enzymes acting on fungal walls

Several plant proteins can act on various fungal wall components. PAGE activity assays of such enzymes are increasingly useful to characterize and follow the fate of these potential antifungal proteins [3].

3.2.2.1. Chitinases

Chitinases (EC 3.2.1.14) are hydrolases of chitin, a β-1,4 polymer of N-acetyl-D-glucosamine residues constituent of fungal walls [77] and invertebrate exoskeletons. Given the importance of chitinases as potential determinants in the resistance of plants to fungal diseases [78,79], PAGE systems were adapted for their analysis.

Detection of chitinase activity after native or

denaturing PAGE is based on the lysis of 0.01% (w/v) glycol chitin (colloïdal chitin or other chitin derivatives) embedded in gels exactly as bacterial walls or suspensions incorporated into gels for assaying bacteriolysis (Section 3.2.1). In the case of chitinolysis, lytic zones after PAGE are revealed by differential staining of intact versus hydrolyzed substrate. Intact chitin and glycol chitin (a water-soluble derivative) can be stained with Calcofluor white M2R as a fluorochrome. After UV transillumination, intact chitin appears highly fluorescent while lysis zones are visualized as dark bands (Fig. 2 and ref. [80]). As for lysozyme PAGE assays, the embedded substrate can be used directly in the denaturing polyacrylamide gel while an overlay substrate-containing gel is required with native polyacrylamide gels (Fig. 2). The PAGE assay of chitinolytic activities involving staining of chitin with Calcofluor white M2R has been applied to several molecular forms of plant chitinases from either normal or stressed plant organs [76,81–86]. For example, up to thirteen chitinase activities have been detected in tobacco leaf tissue infected with tobacco mosaic virus [81].

For better resolution, two-dimensional gel systems may be used to detect individual chitinase forms [81]. The first native PAGE separation is made under either acidic (pH 4.3) or basic (pH 8.9) conditions. The second denaturing (SDS) PAGE is made in non-reducing conditions, since strong reducing agents such as β-mercaptoethanol significantly decrease chitinase activity. An advantage of two-dimensional gels is that the high resolution of proteins allows gels containing glycol chitin to be stained not only with Calcofluor white M2R fluorochrome (UV transillumination) but also with Coomassie Blue followed by aqueous silver nitrate [81]. By such staining, the detection threshold for chitinase activity is increased as compared to the simple detection by Calcofluor [81].

3.2.2.2. Chitin deacetylases

Chitin is an idealized structure since in natural conditions as many as 15 to 20% of the N-acetyl-D-glucosamine residues may be deacetylated to glucosamines. Chitosan, the deacetylated form of chitin, is produced by either a chemical or an enzymatic deacetylation of chitin. In some fungi, chitin deacetylases are responsible for the deacetylation of chitin [87,88].

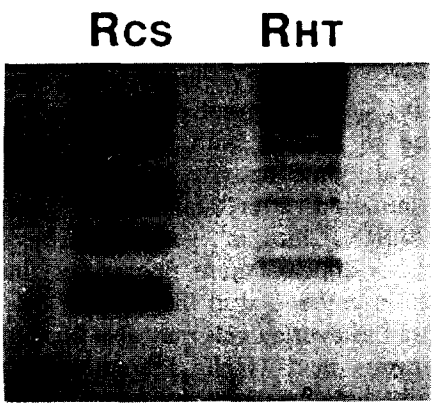

Fig. 2. Electrophoretogram of acidic chitinase activities in root homogenates of cucumber and tobacco. A 40-μl volume (4 μg protein) of root homogenates from 3-day-old cucumber seedlings (R_{CS}) and 6-week-old healthy tobacco plants (R_{HT}) was separated in a 15% (w/v) polyacrylamide slab gel for acidic proteins at pH 8.9 [80]. Homogenates were made (1:1, w/v) in 50 m*M* Tris–HCl (pH 7.5) plus 50 m*M* $CaCl_2$ using a Sorvall Omnimixer and clarified at 15000 *g* for 15 min at 4°C. After electrophoresis, the separating gel was incubated for 5 min at room temperature in 150 m*M* sodium acetate buffer (pH 5.0) and then overlayed with a 7.5% (w/v) polyacrylamide gel containing 0.01% (w/v) glycol chitin as chitinase substrate. The incubation was 1.5 h at 37°C in a closed high humidity plastic box. The overlay gel was stained for 5 min in a solution of 0.01% (w/v) Calcofluor white M2R (syn.: fluorescent brightener 28) in 0.5 *M* Tris–HCl buffer (pH 8.9) and destained in distilled water. Chitinase activities appeared as dark bands against a highly fluorescent background when the stained overlay gel was transilluminated with ultraviolet light [80]. Note that the same type of electrophoretograms and photographs are obtained (dark bands on a fluorescent background) with the chitosanase activity assay when glycol chitosan (chitosanase substrate) is stained with Calcofluor white. The same is also true for other enzyme activity assays involving fluorochrome staining (chitin deacetylase assay with nitrous acid treatment, β-1,3-glucanase assay with fluorochrome staining). Gel assay made by J. Trudel.

A PAGE technique for assaying chitin deacetylases is based on the observation that chitosan is much more fluorescent than chitin after staining with the fluorescent brightener Calcofluor white M2R [89]. The same overall electrophoretic approach is followed as for

lysozymes or chitinases. Proteins are separated by native or denaturing PAGE. For proteins in native gels, a polyacrylamide overlay gel containing 0.1% (w/v) glycol chitin as substrate is incubated in contact with the separation gel. Chitin deacetylase activity is revealed by UV transillumination after staining in Calcofluor white M2R. Chitosan generated by chitin deacetylases appears more fluorescent than the intact chitin embedded in the overlay gel.

Chitosan in overlay gels may also be subjected to a nitrous acid (HNO_2) treatment, which specifically depolymerizes chitosan while leaving chitin intact. Hydrolysis of chitosan by nitrous acid followed by Calcofluor staining yields dark (non-fluorescent) bands (chitin deacetylase activities) in the fluorescent chitin-containing gel [89]. For proteins in denaturing gels, the slab gels contain 0.1% (w/v) glycol chitin as substrate. After SDS-PAGE, enzymes are renaturated in buffered 1% (v/v) purified Triton X-100 [89]. The assays can also be performed in two-dimensional gels [89]. Results with such PAGE assays indicate the presence of several chitin deacetylases in some fungi (Zygomycetes). Up to now, no chitin deacetylase of plant origin has been detected by using such assays [3,89].

3.2.2.3. Chitosanases

Chitosanases hydrolyze chitosan, the deacetylated form of chitin. Occurring naturally in the cell walls of some fungi [90], in terminated growth of rust spores on wheat [91], in pea–*Fusarium* [92] and in gall mite–*Solanum* interactions [93], chitosan elicits various defense reactions in plants [83,94–96].

A simple PAGE assay for chitosanases has been recently used for the study of plant chitosanases [97–99]. Glycol chitosan (a water-soluble chitosan derivative) or chitosan that has to be solubilized in acid is embedded at a final concentration of 0.01% (w/v) into a polyacrylamide gel matrix and used as substrate for chitosanases. As for the preceding enzyme assays, chitosan can be added directly to SDS separating gels or in an overlay gel in contact with native separating gels. It is even possible, however, to add glycol chitosan to native gels run at pH 8.9 [98,99], precluding the use of an overlay gel. Chitosan is stained with Calcofluor white fluorochrome (see previous section on chitin deacetylases). Multiple forms of chitosanase activities were detected by such a PAGE activity assay in lower and higher plant species [99]. Barley, tomato and cucumber chitosanases were further characterized in complementary studies [97]. It is interesting to note that chitosan hydrolysis products can also be easily studied by PAGE analysis [100].

3.2.2.4. β-1,3-Glucanases

Plants produce defense proteins displaying β-1,3-glucanase activity (EC 3.2.1.39) upon β-1,3-glucan substrates such as laminarin or β-1,3-glucans isolated from yeast walls [101]. Laminarin, a reserve oligosacharide isolated from *Laminaria* algae, has been used to detect β-1,3-glucanase (laminarinase) activity after native PAGE by two approaches.

In the first approach, which has allowed study of the expression of pathogenesis-related acidic β-1,3-glucanases of tobacco [102], laminarin (embedded substrate in gels) hydrolysis is revealed by UV fluorescence after Aniline blue [103] or sirofluor [104] staining. In the second approach, laminarin hydrolysis is revealed by the reaction of liberated reducing sugars with triphenyltetrazolium salts [105]. With both techniques, commercial laminarin is the substrate and laminarinase activity is only detected after native PAGE due to a high diffusion of laminarin out of the gel matrix that precludes long incubation periods of proteins in denaturing gels during the renaturation step. Another problem linked to the use of laminarin is the demonstration that some β-1,3-glucanases can act differentially on polymeric insoluble β-1,3-glucans when compared to soluble algal oligomeric laminarin [106].

Alternatively, an alkali-soluble baker's yeast (*Saccharomyces cerevisiae*) β-1,3-glucan [107] was recently used as substrate for electrophoretic detection of β-1,3-glucanase activity after native or denaturing PAGE [101]. The yeast alkali-soluble glucan provides a better detection of most enzymes and, contrary to laminarin, it allows adequate conditions for the detection of

β-1,3-glucanases after SDS-PAGE [101]. Simply, the alkali-soluble yeast glucan is incorporated into gels at a final concentration of 0.6 mg/ml. After PAGE and incubation to allow lysis of fungal glucan, staining of gels is done using Aniline Blue [101]. After destaining in water, lytic zones are revealed by fluorescence using a long-wave UV transilluminator [101].

3.2.2.5. Plant hydrolases acting on cell wall fungi

It is rather easy to detect several plant proteins active on fungal walls by incorporating various fungal wall preparations into gels [97,101]. As a general rule, fungal spores [97] or various cell wall preparations [101] are included into polyacrylamide gels as substrates. The inclusion of fungal spores or cell walls produces an opaque greyish background (as for bacterial walls or suspensions), and lysis is easily revealed by observing clear or opalescent lysis areas corresponding to protein bands in one-dimensional or two-dimensional gels [97,101]. No specific staining is even required. The light transmission contrast between the intact opaque background and the clear lysis zones allows the active proteins to be revealed and photographed against a dark background. Active proteins in gels thus appear as dark active bands on photographs [97,101].

3.2.3. Microbial growth inhibition assays after PAGE

The versatility of the preceding gel activity assays can also be complemented by an overlay assay of microbial growth inhibition after PAGE separation of proteins. Technically, lysozymes, chitinases, chitin deacetylases, β-1,3-glucanases and other microbial cell wall hydrolases can be first assayed in gels for lysis of substrates. In parallel, gels without substrates can also be overlaid with actively growing bacterial or fungal suspensions to determine the capacity of electrophoretically separated proteins to inhibit the growth of microorganisms, especially potential plant pathogens [108,109]. The value of such an approach is that it allows a comparison of antimicrobial lytic activities by using gels overlaid with microbial suspensions and gels run in parallel and embedded with an array of microbial cell wall substrates. The spectrum of substrates is rather wide, varying from either crude cell walls, cells or spore suspensions to specific cell wall fractions [101]. The use of well-characterized microbial wall structures such as those from commercial *Saccharomyces* yeasts [101], notably, is of particular interest since the chemical sequential removal of their cell wall components has been studied in detail [110]. Such an approach, which allows the use of various cell wall preparations enriched in specific cell wall components, recently led to the detection of an M_r 19 000 basic barley protein with a lytic activity against yeast cell walls and an inhibitory effect on the active growth of *Saccharomyces* and *Schizosaccharomyces* yeast fungi [108]. Interestingly, PAGE activity assays involving substrate lysis indicated that this M_r 19 000 barley protein does not exhibit neither laminarinase activity nor other well-known microbial cell wall hydrolase activity [108].

3.2.4. Summary of conditions required to assay microbial cell wall components after PAGE

3.2.4.1. Substrate availability

Many kinds of substrates can be used, varying from microbial cell suspensions to crude cell walls or purified substrates (chitin, chitosan, laminarin, etc.). Two different substrates can even be mixed in gels (chitosan and crude cell walls, for example). The substrate(s) can be included directly in the separation gels or in overlay gels.

3.2.4.2. Activity of proteins after native or denaturing PAGE

Proteins are subjected to native PAGE, denaturing PAGE or 2D polyacrylamide gels. For native PAGE, overlay gels containing the substrate are kept in contact with the separating native gel and an incubation step follows at proper pH and IS. For denaturing gels, the substrate can be incorporated directly in the separating gel. After PAGE, SDS is removed by purified Triton X-100. This allows renaturation

of enzymes. It is important to note that most plant proteins cannot be renatured highly efficiently after SDS denaturation combined with reduction by dithiothreitol (or β-mercaptoethanol). There are, however, defense hydrolytic enzymes that can be denatured and reduced and subsequently renatured rather efficiently. This is the case, notably, with some β-1,3-glucanases [101].

3.2.4.3. Detection of lysis

When the inclusion of the substrate(s) in the gel matrix causes opacity, detection of lysis can be simply made visually. Opacity (greyish) is observed after incorporating microbial cell suspensions, spore suspensions or large amounts of insoluble substrate (such as chitin, for example). When substrates do not produce opacity of gels, staining procedures are required. In fact, differential staining of the intact substrate versus the hydrolyzed substrate is sought. In many cases, polymeric intact substrates can react with fluorochromes allowing UV transillumination as visualization and photography procedures. In other cases, commonly used non-highly-specific stains can be also useful. The best example is with chitosan as a substrate for chitosanases, where amino residues are highly reactive to Coomassie Blue (R-250 or G-250) staining. This staining is even enhanced when including a final aqueous silver nitrate step.

3.2.4.4. Concluding remark

Imagination is possibly the most important limit to the use of substrate(s) embedded in gels. All kinds of cell, cell wall preparations, cell components and various chemicals can potentially be used as substrates. Suspensions of nematode eggs, microbial exopolysaccharides and mucilages represent a few examples of potential substrates of diverse enzymes. The same is true for pollen grains and spores of all kinds that can be used as substrates. In many cases, it is not even necessary to look for specifically staining the embedded substrate. Its presence in the gel matrix is simply revealed by opacity to the naked eye. Several applications of this simple approach will surely be found in the near future. Since the first PAGE assay of chitinases in 1989, it is comforting to note that chitinase PAGE assays have been used in fields as diverse as medicine [111,112], animal biology [113], entomopathology [114], mycology [115,116], plant biology [117–120] and phytopathology [121–126].

3.3. Studying defense responses/resistance to pests

Concurrently with the development of hydrolytic enzymes contributing to limit microbial invasions, plants have developed defense proteins intended to minimize wounding by herbivore pests. Rather than hydrolyzing cell wall constituents of target organisms, these proteins act as anti-nutritive substances interfering with either protein or carbohydrate digestion [127,128].

3.3.1. Proteins interfering with protein digestion

In adverse conditions, plants systemically produce PIs that strongly inactivate digestive proteinases and limit the growth of various insect pests [127]. When DNA sequences encoding these inhibitors are transferred into plant genomes, PIs also represent efficient biocontrol compounds for controlling specific pests [129]. For such a control approach, however, it is important to consider whether the exogenous PIs produced in the plants may also interfere with plant endogenous proteinases, which represent potential target enzymes for these inhibitors [39]. To assess correctly the potential of PIs, gel electrophoretic assays allowing spatial and temporal distribution of plant proteases should thus be performed in parallel with studies on the potentially useful inhibitors.

3.3.1.1. Proteases

Several useful techniques have been developed for the analysis of proteinases using PAGE systems [130]. In particular, electrophoresis of proteolytic enzymes on acrylamide gels containing an appropriate high-molecular-mass substrate entrapped in the gel has been explored as a general method of proteinase resolution and

detection [131,132]. The rapid and sensitive method developed by Heussen and Dowdle [131] uses gelatin-containing polyacrylamide gels to separate proteins under weakly-denaturing conditions. After renaturation with Triton X-100, the active proteinases leave clear bands in the gel once it is stained with Coomassie Brilliant Blue. This system, primarily employed to study human proteinases, has recently been adapted to study proteinases of either microbial [133–136] and plant [39,58,137] origin. Highly sensitive for the detection of plant proteinases [58], this method is useful for the analysis of multiple proteolytic patterns characterizing various extracts.

When used in conjunction with class-specific PIs, it also allows discrimination between the different classes of proteinases separated on gels [58,133,134]. After electrophoresis, low-molecular-mass PIs such as EDTA, PMSF or leupeptin are added to the buffer during proteolysis [133]. When staining the gel, the protease bands corresponding to the class of the PIs used are weak (or absent) as compared to non-inhibited control bands. Rather than performing a post-electrophoretic inhibition, a pre-electrophoretic inhibition may also be used [58]. In this case, irreversible PIs such as PMSF and E-64 are used to discriminate between proteinase classes (Fig. 3). When using this pre-migration approach, all risks of non-controlled proteolysis during migration are prevented, both inhibited and non-inhibited extracts may be studied on the same gel, and the quantity of inhibitors used is minimal [58]. It is also of interest to mention that the inhibiting effects of PMSF and E-64 are maintained for electrophoresis conducted in the absence of SDS. The use of these PIs may thus be useful to determine the class of proteinases that are difficult to renature following SDS treatment. Although proteinases from maize seed extracts have been correctly resolved [138], the proteinases separated in native conditions conserve their native form and hydrolysis of gelatin may occur during migration, resulting in a much lower resolution of proteinases on native gels as compared to SDS-PAGE systems. By preventing all risk of uncontrolled proteolysis during migration, two-step procedures involving notably the use of gelatin-containing overlay gels [139], may be more appropriate in some cases.

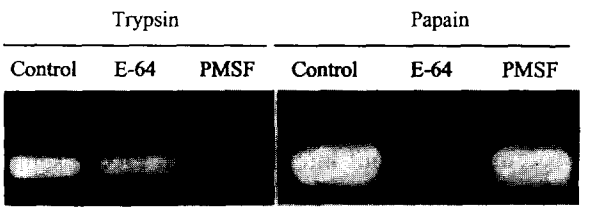

Fig. 3. Electrophoretogram showing gelatinolytic activity of trypsin and papain preincubated with class-specific proteinase inhibitors. The enzymes were dissolved at a concentration of 0.25 mg/ml (10 μM) in water containing 0.1% (v/v) Triton X-100, incubated with either E-64 (a cysteine PI) or PMSF (a serine PI), and submitted to 0.1% (w/v) gelatin/10% (w/v) SDS-PAGE [58]. After migration, the gel was transferred to a 2.5% (v/v) Triton X-100 aqueous solution for 30 min at room temperature, placed in an activation buffer (50 mM phosphate, pH 6.8, 5 mM EDTA, 5 mM L-cysteine, 0.1% (v/v) Triton X-100) for 45 min at 37°C, and finally stained with Coomassie Brilliant Blue [58]. Non-inhibited proteolytic activities appeared as transparent zones against a blue background corresponding to non-hydrolyzed gelatin. Control wells correspond to enzyme preparations preincubated with water containing 0.1% (v/v) Triton X-100.

For both denaturing and native systems, it is also important to mention that gelatin interferes with proteinase migration [58]. Varying the gelatin level in gels from 0.1 to 0.4% (w/v), for instance, has a linear effect on the relative migration of the two serine proteinases from tomato fruit [58]. Similarly, the distance separating the two bands decreases by about 30% with an increase in gelatin from 0.1 to 0.4% (w/v). It cannot be determined yet whether this retardation effect is an active one (chemical interference due to an affinity-binding process) or a passive one (physical interference due to a network effect), but it is important to keep in mind that this drop in resolving power could result in the underestimation of the number of proteolytic enzymes in an extract and thus lead to erroneous conclusions. In addition, given the interference observed, determination of molecular masses using gelatin-PAGE may not be appropriate. The plant cysteine proteinase papain, for instance, migrates to an apparent M_r of about 75 000 in 0.1% (w/v) gelatin-polyacrylamide gels, while its theoretical M_r is 23 400 [58]. When

estimations of molecular masses are required, alternative strategies involving two-step procedures should be used. A blotting system using gelatin as a substrate fixed onto a nitrocellulose sheet, notably, allows a sensitive detection of proteinases without interfering with PAGE separation of proteinases [140].

3.3.1.2. Protease inhibitors

An efficient way to detect plant PI activity is to perform a PAGE separation of PIs followed by a hydrolysis step with specific proteinases [141,142]. Briefly, this method consists of separating proteins of an extract suspected to contain PIs using a gelatin–polyacrylamide gel. After migration, the gel is incubated in a buffer containing one or several proteinases. While gelatin and other non-PI proteins in the gel are completely hydrolyzed, bands corresponding to class-specific inhibitors of the proteinase(s) used are not degraded and leave blue bands in the gel once it is stained with Coomassie Brilliant Blue [141,142]. Such "reverse activity gels" may prove useful when searching for class-specific PI activity in crude extracts or when studying activity of PIs structurally modified via genetic engineering. By using such gels, for instance, the activity of rice cystatin, a cysteine PI potentially useful for the control of several Coleoptera [143,144], was shown to be maintained even when an M_r 26 000 polypeptide such as glutathione S-transferase is linked to its NH_2-terminal extremity, demonstrating the remarkable conformational stability of this inhibitor (Fig. 4 and Ref. [142]).

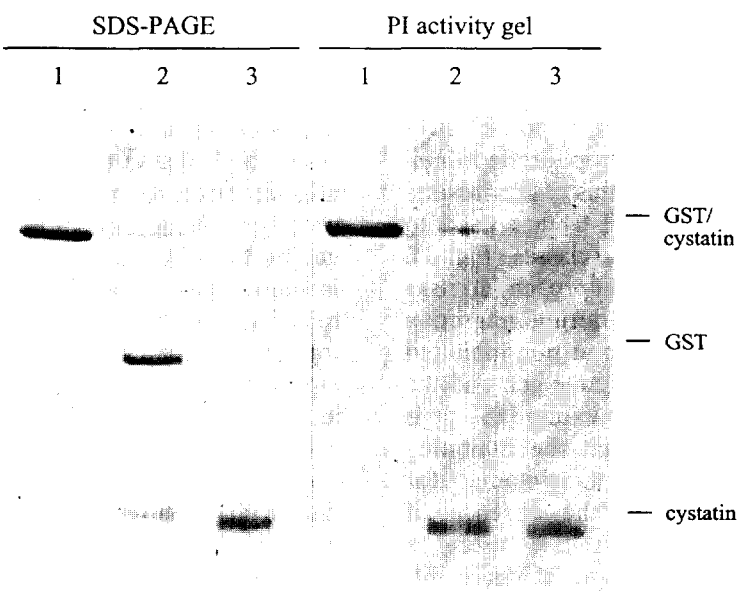

Fig. 4. Electrophoretogram showing SDS-PAGE migration and activity of rice cystatin expressed in *Escherichia coli* as a glutathione S-transferase (GST) fusion protein. Purity of the protein products was verified by 12% (w/v) SDS-PAGE. Activity of the cysteine PI, either isolated from or linked to GST, was detected on gel by 0.1% (w/v) gelatin-SDS-PAGE [58] followed by proteolysis with papain [141,142]. Briefly, inhibitor-containing extracts were first submitted to weakly-denaturing 0.1% (w/v) gelatin/SDS-PAGE [58]. After migration and renaturation with Triton X-100 (see legend of Fig. 3), the gels were placed in a proteolysis buffer (50 m*M* phosphate, pH 6.8, 5 m*M* EDTA, 5 m*M* L-cysteine, 0.1% (v/v) Triton X-100) containing papain (250 μg/ml buffer) for 4 h at 37°C, and finally stained with Coomassie Brilliant Blue [142]. Cysteine PI activities were visualized as blue bands corresponding to areas non hydrolyzed by the cysteine proteinase papain. Note the activity of the fusion protein in lane 1, which indicates the high conformational stability of rice cystatin. Lanes: 1 = GST/cystatin fusion protein (M_r 43 000) purified from *E. coli* ; 2 = purified GST/cystatin after cleavage with a specific protease; 3 = rice cystatin (M_r 17 000) recovered after cleavage of the fusion protein.

3.3.2. Proteins interfering with carbohydrate digestion

Apart from inhibitors of proteinases, plants produce inhibitors of starch hydrolysis (amylase inhibitors: AIs) that may serve as a protection against insect pests either in natural or transgenic systems [145]. As for PIs, the possible inactivation of plant amylases by exogenous AIs must be considered and renders necessary the analysis of endogenous α- and β-amylase forms. Several gel electrophoretic techniques have been proposed to analyze amylase activity on gels (see Ref. [52] for a review). To our knowledge, no procedure was yet reported for the specific detection of AI activity using gel electrophoresis. The development of procedures similar to that reported for PIs (Section 3.3.1) may possibly enable such studies.

4. Conclusions

The numerous reports published in recent years on this topic clearly indicate the importance of gel electrophoresis for the study of plant proteins. Several decades after its emergence, this technique remains one of the most versatile tools to study the various biochemical and biophysical characters of plant proteins. Recent technical advances such as genetic engineering and fluorescence microscopy, rather than causing its decline, simply contribute to confirm its importance. The understanding of specific physiological or defense processes via genetic transformation, for instance, will be allowed to the extent that efficient and adapted procedures will be available to study the recombinant proteins produced. Finally, the elucidation of complex biological processes partially depends on the capacity to separate and detect proteins, which are always key components of these processes.

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