Characterization of two membrane-associated β -glucosidases from maize (*Zea mays* L.) coleoptiles

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We isolated membrane vesicles from maize (*Zea mays* L.) coleoptiles and identified in these vesicles a 58 kDa (pm58) and a 60 kDa (pm60) protein by photoaffinity labelling with 5-azido-[7-³H]indole-3-acetic acid ([³H]N₃IAA). Photoaffinity labelling was effectively competed for by auxins as well as by flavonoids. The labelled proteins were solubilized by Triton X-114 from the vesicles and partially purified. Microsequence analysis revealed that pm60 is a β -glucosidase. This was confirmed by biochemical and immunological analysis. We show that pm60 has a β -D-

glucoside glucohydrolase (EC 3.2.1.21) activity. It uses *p*-nitrophenyl β -D-glucopyranoside (PNPG) as a substrate, with a pH optimum of 5.0. The K_m for PNPG is 0.652 mM and the V_{max} 6.24 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$. The β -glucosidase activity of pm60 was competitively inhibited by IAA and 1-naphthylacetic acid as well as by gluconolactam and glucose. N-terminal amino-acid-sequence analysis of pm58 revealed similarity to pm60, suggesting that both proteins are encoded by different members of a gene family.

INTRODUCTION

β -Glucosidases (β -D-glucoside glucohydrolases; EC 3.2.1.21) catalyse the hydrolysis of aryl and alkyl β -D-glycosides and were among the first enzymes to be studied (Liebig and Wöhler, 1837). A wide variety of plant glycosides are known, including glycosidic conjugates of phenolic compounds such as flavonoids or anthocyanins, cyanogenic glycosides, steroids, plant growth regulators, as well as a large number of conjugates of secondary metabolites (for an overview, see Hösel & Conn, 1982). Whereas glucosyl-transferases catalyse the conjugation of such plant metabolites with sugar nucleotides (mostly UDP-glucose), resulting in their inactivation, accumulation and storage as glycosides within plant cells, the glucosidases hydrolyse such glycosides, thereby releasing the biologically active compounds from the inactive conjugates.

Conjugation of phytohormones to low-molecular-mass substances is well known and thought to play an important role in phytohormone metabolism and probably in the homeostatic control of hormone concentrations (see Palme and Schell, 1993). Recent discoveries have illustrated the important role of β -glucosidases in the control of phytohormone concentrations. Several plant pathogens encode enzymes that either conjugate phytohormones or hydrolyse phytohormone conjugates (Kosuge et al., 1983; Estruch et al., 1991a,b). The *rolB* and *rolC* oncogenes from *Agrobacterium rhizogenes*, for example, were shown to encode proteins with β -glucosidase activities which *in vitro* hydrolyse indoxyl *O*-glucoside and cytokinin glucosides respectively (Estruch et al., 1991a,b). Using 5-azido-[7-³H]indole-3-acetic acid ([³H]N₃IAA), a 60 kDa β -glucosidase that hydrolysed indoxyl *O*-glucoside and cytokinin glucosides *in vitro* has recently been identified in cytosolic extracts from maize (*Zea mays* L.) coleoptiles (Campos et al., 1992; Brzobohaty et al., 1993). Here we report the identification of two proteins, termed pm58 and pm60, in highly purified membrane vesicles from maize coleoptiles. We partially purified both proteins and present evidence

from amino acid analysis and *in vitro* biochemical assays that these proteins are β -glucosidases.

MATERIALS AND METHODS

Radiochemicals and chemicals

5-N₃-[7-³H]IAA (21.7 Ci/mmol; 1 Ci = 37 GBq), was synthesized as described by Campos et al. (1991). All other chemicals were of analytical grade and purchased from Sigma (München, Germany) or Merck (Darmstadt, Germany) if not stated otherwise.

Plant material

Seeds of *Zea mays* L. (cv. mutin 240; Kleinwanzlebener Saatzeit, Einbeck, Germany), were soaked in water overnight and grown on moist cotton for 3 days at 28 °C in the dark. Coleoptiles, including the primary leaf, were harvested, frozen in liquid N₂ and stored at –70 °C.

Preparation of membranes

The following procedures were performed at 4 °C. Coleoptiles (200 g), including the primary leaves, were homogenized with a Waring Blendor and a Polytron homogenizer (at setting 7–8) in buffer 1 [100 mM Tris/citric acid (pH 8.0)/500 mM sucrose/1 mM EDTA/5 mM ascorbic acid/0.1 mM MgCl₂/0.6 polyvinylpyrrolidone/1 mM dithiothreitol/3.5 mg/l aprotinin/0.1 $\mu\text{g}/\text{ml}$ leupeptin 2.5 ml/g fresh weight). The homogenate was filtered through a nylon net (135 μm pore size) and the particulate material retained in the net was re-extracted (1:1, v/v). The combined homogenates were centrifuged for 10 min at 10000 *g*. A microsomal pellet was obtained by centrifugation at 142000 *g* for 40 min. The microsomal pellet was suspended in buffer 2 (330 mM sucrose/5 mM potassium phosphate, pH 7.8) and adjusted to 9 g. Membrane vesicles were isolated by two-phase

Abbreviations used: IAA, indole-3-acetic acid; [³H]N₃IAA, 5-azido-[7-³H]IAA; 1-NAA, 1-naphthylacetic acid; NPA, 1-N-naphthylphthalamic acid; PNPG, *p*-nitrophenyl β -D-glucopyranoside; PVDF, poly(vinylidene difluoride).

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partitioning using dextran T-500 and poly(ethylene glycol) 3350 (Feldwisch et al., 1992). The resulting membrane vesicles were resuspended in 0.5 ml of buffer 3 [10 mM Tris/Mes (pH 6.5)/250 mM sucrose]. Aliquots were frozen in liquid N₂ and stored at -70 °C.

Photoaffinity labelling

Photoaffinity labelling was performed as described by Campos et al. (1991). Samples containing 100 µg of protein were incubated in buffer 3 containing 1 µM [³H]N₃IAA for 10 min on ice. Optionally, unlabelled auxins and related compounds were added for competition analysis. Photolysis was performed at -196 °C for 10 min, using a high-pressure mercury lamp (Philips HKP 125 W/L). After illumination, membranes were recovered by centrifugation at 108 000 g for 11 min. Pellets were dissolved in SDS loading buffer, heated to 70 °C for 5 min, and separated by SDS/PAGE (12.5% gel) (Laemmli, 1970). Fluorography was performed as described by Skinner and Griswold (1983). Dried gels were exposed for 1 day to preflashed Kodak XAR-5 film.

Partial purification of pm60 and pm58

Membrane vesicles containing 300 µg of protein were isolated, resuspended in 500 µl of buffer 3 containing 1% (v/v) Triton X-100 and incubated on a shaker at 4 °C for 30 min. After centrifugation at 2 °C at 108 000 g for 15 min, pellets were dissolved in 500 µl of buffer 4 [10 mM Tris/HCl (pH 7.5) 150 mM NaCl/1% (v/v) Triton X-114] and shaken at 4 °C for 30 min. Samples were subsequently incubated at 37 °C for 15 min, followed by centrifugation at 2000 g for 5 min to facilitate phase separation (Bordier, 1981). Proteins in the Triton X-114-enriched lower phase were precipitated with 10% (v/v) trichloroacetic acid for 2 h on ice and analysed by SDS/PAGE.

Protein sequence analysis

Proteins separated by SDS/PAGE were electroblotted on to poly(vinylidene difluoride) (PVDF) membranes (Millipore, Eschborn, Germany) in 50 mM Tris/50 mM boric acid, pH 8.3. Proteins were revealed after staining for 40 s with Amido Black [0.1% (w/v) in 45% (v/v) methanol (h.p.l.c. grade)/water] and destaining with water. For trypsin digestion, bands were excised from the PVDF membrane and incubated in 100 mM Tris/HCl, pH 8, with 1 µg of trypsin (Boehringer, Mannheim, Germany) for 4 h at 37 °C. Tryptic peptides were recovered after washing the excised PVDF pieces once with 100 µl of 80% (v/v) formic acid, four times with 100 µl of water, and then separated on a VYDAC C4 RP column (4.6 mm × 250 mm). Peptides were eluted at a flow rate of 0.4 ml/min with a linear gradient ranging from 0 to 37% solvent B in solvent A for 60 min, 37–75% B for 32 min, and 75–100% B for 10 min. Solvent A was aq. 0.06% trifluoroacetic acid and solvent B was 80% (v/v) acetonitrile in aq. 0.056% trifluoroacetic acid. Peptides were detected at 214 nm using a diode-array detector to allow peak purity analysis [Hewlett-Packard HP-1090 (Waldbronn, Germany) HP-1090 h.p.l.c. instrument]. Peptides were collected by hand and used directly for sequence analysis by automated Edman degradation in an Applied Biosystems 477A sequenator equipped with an on-line phenylthiohydantoin amino acid analyser (model 120A). Both sequenator and analyser were operated as described by the manufacturer. For N-terminal sequence analysis, protein bands were excised from the PVDF membrane and mounted directly in the reaction chamber of the protein sequenator.

Enzyme assay

β-Glucosidase activity was analysed by measuring the amount of *p*-nitrophenol released from *p*-nitrophenyl β-D-glucopyranoside (PNPG). Each assay was performed in triplicate in a total volume of 300 µl. Membrane vesicles containing 0.2–10 µg of protein were incubated in assay buffer [30 mM sodium citrate/citrate acid (pH 5.0)/5 mM MgCl₂] with 0.08–5.0 mM PNPG for 15 min at 30 °C. The reaction was stopped by addition of 500 µl of stop solution (2 M Na₂CO₃). The absorbance of released *p*-nitrophenol was measured at 405 nm using a spectrophotometer. A molar absorption coefficient of 17.5 ml·µmol⁻¹·cm⁻¹ was used to calculate the amount of *p*-nitrophenol released (John and Schmidt, 1985).

Immunological techniques

Polyclonal antisera were raised in rabbits against recombinant p60 (L. Bako, B. Brzobohaty and K. Palme, unpublished work) as described by Catty and Raykundalia (1988). Sera were affinity-purified as described by Harlow and Lane (1988). For immunostaining of Western blots, a secondary goat anti-rabbit antibody coupled to alkaline phosphatase was used as described by Blake et al. (1984). For immunoprecipitation, affinity-purified antibodies were preincubated with Protein A beads for 1 h at 4 °C and added to a protein fraction solubilized from membrane vesicles with Triton X-114. Immunoprecipitation was performed in a total volume of 120 µl for 1.5 h at 4 °C using a 1:1 ratio of Triton X-114-solubilized proteins from membrane vesicles and IgG (0.5 µg of protein each). After centrifugation, 100 µl of the supernatant was used for β-glucosidase assays.

Purification of p58

Preparation of membranes and extraction with butan-1-ol was as described by Campos et al. (1992). The crude membrane extract was purified on CM-Sepharose equilibrated with 10 mM Tris/Mes (pH 6.5)/5 mM MgCl₂. In the 50 mM NaCl eluate, p58 was identified by photoaffinity-labelling with [³H]N₃IAA. The eluate was concentrated on a small Q-Sepharose column (1 ml gel bed volume) and used for protein-sequence analysis.

RESULTS

Photoaffinity labelling of pm58 and pm60 with [³H]N₃IAA is competed for by IAA

Membrane vesicles were isolated from maize coleoptiles by two-phase partitioning and used for photoaffinity labelling with 1 µM [³H]N₃IAA at -196 °C. Two polypeptides with molecular masses of 60 kDa (pm60) and 58 kDa (pm58) were detected after fluorography for 1 day (Figure 1). A 20-fold reduction of the concentration of the labelling reagent to 0.05 µM resulted in 10-fold longer exposure times. Photoaffinity labelling of both proteins was reduced by addition of unlabelled IAA to the reaction (about 40% reduction of labelling), as well as tryptophan (about 10–20%). However, photoaffinity labelling was not affected by 2,4-dichlorophenoxyacetic acid, benzoic acid and *p*-aminobenzoic acid as a scavenger (results not shown). The auxin-transport inhibitor 1-naphthylphthalamic acid (NPA) slightly reduced the labelling intensity (10–20%), while no reduction was observed after addition of 2,3,5-triiodobenzoic acid. In addition, we have used flavonoids as competitors known to compete with binding of [³H]NPA to its putative receptor and which perturb auxin transport in a variety of plant tissues (Jacobs and Rubery, 1988). In contrast with NPA, quercetin and kaempferol reduced

Figure 1 Photoaffinity labelling of pm60 and pm58 with [3 H] N_3 IAA and competition with flavonoids and flavonoid glucosides

Membrane vesicles containing 100 μ g protein were incubated with 1 μ M [3 H] N_3 IAA for 10 min at pH 6.5 with or without 1 mM competitor. After illumination at -196°C , proteins were separated by SDS/PAGE. Fluorography was for 1 day at -70°C using a preflashed Kodak X-Omat AR-5 film. Locations of molecular-mass (M) markers are indicated. Lane a, control without competitor; lane b, quercetin; lane c, rutin; lane d, kaempferol.

Figure 2 Solubilization of pm60 and pm58 from membranes

Photoaffinity-labelled membrane vesicles were extracted either with Triton X-100 (TX-100), Triton X-114 (TX-114), acetone or butan-1-ol as described in the Materials and methods section. Solubilized proteins (S) were separated from insoluble proteins (P) by ultracentrifugation. Proteins were analysed by SDS/PAGE and fluorography. T, Triton X-114 phase, I, interphase.

the [3 H] N_3 IAA labelling slightly (Figure 1, lanes b and d), whereas rutin, a quercetin β -D-glucoside, inhibited the photoaffinity labelling of pm60 almost completely (Figure 1, lane c), but did not influence pm58 labelling.

Solubilization of photoaffinity-labelled proteins

We tested detergents (Tritons X-100 and -114) and organic solvents (acetone and butan-1-ol) for their ability to solubilize pm58 and pm60 from the membrane vesicles. After incubation of the membranes with 1% Triton X-100 and acetone, we found both proteins in the insoluble membrane pellet, whereas, after butan-1-ol extraction, proteins were found in the insoluble

Table 1 Protein sequence analysis of pm60 and pm58

Peptides of pm60 were produced by *in situ* digestion of proteins blotted on to PVDF, and they were sequenced by automated Edman degradation as described in the Materials and methods section. Amino acid differences between pm60 and pm58 are indicated by a shaded box.

Amino acid no. ...	Sequence									
	1	5	10	15	20					
pm60 N-terminus...	S	A	V	G	Q	N	G	V	Q	M
Tryptic peptide no.	L	S	P	S	E	I	P	Q		
3a	W	L	K							
3b	?	M	?	M	?	A	Y	R		
8	E	M	G	M	D	A	Y	R		
11a	Y	G	I	V	Y	V	D	R		
11b	V	G	S	Q	N	G	V	Q	M	L
12	I	L	D	G	S	N	S	D	I	G
19a	Y	G	N	P	P	I	Y	I	T	E
19b	S	I	V	E	D	Y	T	Y	F	A
26	L	A	G	S	Y	N	M	L	G	L
29	?	?	P	G	L	D	?	A	Y	P
33	N	?	L	T	F	N	E	P	Q	?
pm58 N-terminus...	S	A	V	G	Q	N	G	V	Q	

interphase (Figure 2). Incubation of membrane vesicles with 1% Triton X-114, however, efficiently solubilized pm58 and pm60. Both proteins were found in the hydrophobic, Triton X-114-enriched phase. Only a small portion (less than 5%) was found in the aqueous Triton X-114-depleted phase (Figure 2; compare TX-114 T and S).

Partial purification and protein-sequence analysis

Membrane vesicle were obtained by two-phase partitioning and extracted with Triton X-100. The Triton X-100-insoluble membrane residue was re-extracted with Triton X-114 as described by Bordier (1981). We observed an enrichment of pm58 and pm60 to about 1% and 30% of the total protein content in the Triton X-114 phase respectively (Figure 3a). Proteins within the Triton X-114 phase were precipitated with trichloroacetic acid, separated by SDS/PAGE and electroblotted on PVDF. N-terminal amino acid sequences of pm58 and pm60 were determined directly on Amido Black-stained PVDF (see Table 1). To obtain additional amino-acid-sequence information, bands corresponding to pm60 were excised from PVDF and digested with trypsin. Peptides were separated by reversed-phase chromatography on a VYDAC C4 column (Figure 3b). The numbered peaks were used for sequence analysis by automated Edman degradation (Figure 3b; Table 1). The sequence of peptide 11b corresponded to part of the pm60 N-terminus. We determined the N-terminal amino acid sequence of pm58 and found that amino acid residues 3 (Tyr/Arg) and 6 (Asn/Ser) differed from pm60, whereas all other residues were identical (Table 1). Comparison of the amino acid sequences obtained for pm60 with the PIR protein database suggested that pm60 is a β -glucosidase, as peptides 8, 11a, 12, 19 and 26 were highly similar to various β -D-1,4-glucosidases from *Caldocellum saccharolyticum* (Love et al., 1988), *Bacillus polymyxa* (Gonzalez-Candelas et al., 1990), *Candida pelliculosa* (Kohchi and Toh-e, 1985). The N-terminal amino acid sequence of pm60, however, was completely identical

Figure 3 Partial purification of pm60 and pm58 and separation of pm60 tryptic peptides

(a) Membrane vesicles containing 200 μ g of protein were extracted successively with Triton X-100 and Triton X-114. Proteins in the lower, Triton X-114-enriched, phase were precipitated with 10% trichloroacetic acid, separated by SDS/PAGE, electroblotted to PVDF membrane and stained with Amido Black. Arrowheads indicate pm60 and pm58. The locations of molecular-mass (*M*) markers are indicated. (b) Two bands corresponding to pm60 were excised from PVDF and digested with trypsin. Peptides were separated by reversed-phase chromatography on a VYDAC C4 column. The sequences of numbered peaks are given in Table 1.

Table 2 Protein sequence analysis of pm58

p58 was partially purified as described in the Materials and methods section. Tryptic peptides were produced by *in situ* digestion of proteins blotted on to PVDF, and they were sequenced by automated Edman degradation as described in the Materials and methods section. Amino acids which differ from the sequence of p60 are indicated by a shaded box.

Sequence					
Amino acid no. ... Peptide no.	1	5	10	15	20
8	I	D	I	S	P
12	E	M	G	M	D A Y R
14	Y	G	G	F	L D K
15	I	M	?	G S N	A ? I G A N S Y H M Y K
17	?	?	I	V	Y V I M Q
19	G	?	Y	P	F S M
20	V	P	Y	G T S	F L D E Q A
22	E	?	L	P	F F E N R

with the sequence of a 60 kDa β -glucosidase from maize (Esen, 1992; Brzobohaty et al., 1993).

In addition we purified a 58 kDa protein (p58) from a crude membrane preparation which, like p60, was solubilized with acetone, butan-1-ol and Triton X-100. The purification scheme of p58 differed from p60 in the way in which it bound to CM-Sepharose. p58 was retained by CM-Sepharose, whereas p60 was found in the flow-through (Campos et al., 1992). The amino acid sequences of tryptic peptides of p58 are listed in Table 2. Again, like the N-terminus of pm58, the amino acid sequences of p58 peptides were very similar to, but not identical with, the amino

acid sequence of p60 (sequence identities of p58 peptides to p60 were in the range of 55–100%). Both the different binding characteristics to CM-Sepharose and the amino acid sequence differences indicate that p58/pm58 is not a degradation product of p60/pm60, as suggested by Esen (1992).

pm60 has β -glucosidase activity

The amino acid sequence similarity of pm60 to β -glucosidases prompted us to assay for a pm60-related β -glucosidase activity. The enzymic activity was analysed by measuring the amount of *p*-nitrophenol released from PNPG. We observed that the membrane-associated β -glucosidase activity was linearly dependent on the protein concentration over the range 0.5–10 μ g. The pH optimum was at pH 5.0. The kinetic constants (K_m and V_{max}) were determined by the use of increasing concentrations of PNPG. Using the computer program ENZFITTER (Elsevier-Biosoft) for data analysis, we obtained a K_m of 0.652 mM and a V_{max} of 6.24 μ mol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$ for PNPG. We analysed the β -glucosidase activity during the extraction of plasma membranes with Triton X-100 and Triton X-114. We found that the specific activity for PNPG hydrolysis increased 5-fold in the Triton X-114-enriched phase after extraction of membrane vesicles (β -glucosidase activity in membrane vesicles: 5.4 μ mol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$; Triton X-114 phase: 24.53 μ mol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$). A significant decrease of the β -glucosidase activity to 0.32 μ mol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$, however, was detected in the protein fraction solubilized with Triton X-100. We analysed the substrate specificity of the membrane-associated β -glucosidase. The data summarized in Table 3 show that neither *p*-nitrophenyl α -D-glucopyranoside, *p*-nitrophenyl β -D-galactopyranoside, *p*-nitrophenyl β -D-xylopyranoside nor *p*-nitrophenyl α -D-arabinofuranoside were hydrolysed, suggesting that the enzyme is relatively specific for aryl- β -D-linked glucopyranoside substrates.

To test whether the activity measured in our experiments was due to pm60, we analysed pm60-specific antibodies in immuno-

Table 3 Substrate specificity of the membrane-associated β -glucosidase

Membrane vesicles containing 0.5 μ g of protein were incubated with one of several *p*-nitrophenyl (PNP) substrates at 4.2 mM for 15 min at 30 °C. Released *p*-nitrophenol was determined at 405 nm.

PNP substrate	Specific activity (μ mol $\cdot$ min ⁻¹ $\cdot$ mg ⁻¹)
β -Glucopyranoside	5.4
α -Glucopyranoside	0.027
β -Galactopyranoside	0.693
β -Xylopyranoside	0.069
α -Arabinofuranoside	0.182

Figure 4 Western-blot analysis of membrane vesicles and analysis of pm60-specific β -glucosidase activity: detection of pm60 in membrane vesicles by affinity-purified IgG

The arrowhead points to alkaline phosphatase-stained pm60. *M*, molecular mass.

competition experiments. As shown in Figure 4, our affinity-purified antibody recognized pm60 in a Western blot containing proteins from maize coleoptile membrane vesicles obtained after two-phase partitioning. After immunoprecipitation of pm60 from Triton X-114-solubilized membranes, the residual β -glucosidase activity decreased in several independently performed experiments to between 5.8 and 8.9 %, indicating that the β -glucosidase activity assayed was due to the presence of pm60.

pm60 β -glucosidase activity is influenced by auxins

pm60 was identified by photoaffinity labelling with [³H]N₃IAA. We therefore tested whether the pm60 β -glucosidase activity was affected by auxins. We incubated membrane vesicles with 0.666 mM PNPG and 1 mM of either IAA, 1-NAA or benzoic acid. The enzymic activity was reduced to 20 % by 1 mM 1-NAA and 45 % by 1 mM IAA, but not with benzoic acid. For further characterization we determined the kinetic constants, *K_i*, for 1-

Figure 5 Competitive inhibition of the membrane-associated β -glucosidase with 1-NAA and IAA

Membrane vesicles containing 0.5 μ g of protein were mixed with increasing concentrations of PNPG (0.083–8.3 mM) and incubated for 15 min at 30 °C with and without the indicated concentrations of either 1-NAA (a) or IAA (b). Released *p*-nitrophenol was determined and data analysed with the computer program ENZFITTER (Elsevier–Biosoft). The graphs show Lineweaver–Burk plots.

Table 4 *K_i* values for competitive inhibitors of m60-specific β -glucosidase activity

Membrane vesicles containing 0.5 μ g of protein were assayed as described in Figure 5 with several inhibitors. Data were analysed with the computer program ENZFITTER (Elsevier–Biosoft).

Inhibitor	<i>K_i</i> (mM)
IAA	1.138
1-NAA	0.432
Glucono-Lactam	0.839
Glucose (0.1 M)	148.06

NAA and IAA as well as for D-gluconolactone and glucose, well-known competitive inhibitors of β -glucosidase. As shown in Figure 5, 1-NAA and IAA are competitive inhibitors of the membrane-associated pm60 specific β -glucosidase. The *K_i* values for 1-NAA, IAA, D-gluconolactone and glucose are listed in Table 4.

DISCUSSION

The photoactivatable synthetic auxin analogue [³H]N₃IAA has been successfully used by several laboratories to identify auxin-binding proteins (Hicks et al., 1989a,b; Jones and Venis, 1989; Macdonald et al., 1991; Feldwisch et al., 1992; Zettl et al., 1994;

Bilang et al., 1993). Auxin-binding proteins are thought to be involved in various aspects of auxin action. Among the proteins recently identified by [^3H]N $_3$ IAA labelling in highly purified membrane fractions was a glutathione S-transferase that may be involved in detoxification of hormones (Zettl et al., 1994). Another enzyme identified by photoaffinity labelling with [^3H]N $_3$ IAA was a 60 kDa protein (p60), which was primarily found in the cytosolic fraction of maize coleoptiles (Campos et al., 1992). A small fraction of this protein, however, was also found in the microsomal pellet, from which it could be released by acetone, butan-1-ol or Triton X-100. p60 purified from maize coleoptiles, as well as recombinant p60, was shown by *in vitro* biochemical assays to have β -glucosidase activity (Campos et al., 1992; Brzobohaty et al., 1993).

In highly purified membrane vesicles from maize we have identified, by photoaffinity labelling with [^3H]N $_3$ IAA, two proteins termed pm58 and pm60 according to their molecular masses. We were able to solubilize pm58 and pm60 with Triton X-114, but not with acetone, butan-1-ol or Triton X-100. This markedly contrasts with the solubilization of p60, which was readily solubilized by either acetone, butan-1-ol or Triton X-100, suggesting that pm58 and pm60 are probably tightly associated with the membranes.

Partial amino acid sequence analysis of pm60 revealed that its N-terminus was identical, over a stretch of 20 amino acid residues, with that of p60 (Brzobohaty et al., 1993; Esen, 1992). Interestingly, the membrane-associated pm60 has a five-residue long N-terminal extension, suggesting different processing of the pm60 and the p60 protein. Peptide sequences of pm60 were completely identical with the amino acid sequence of p60. Thus pm60 and p60 appear to represent two forms of maize β -glucosidase, with different solubilities, from membranes. The N-terminal amino acid sequence of pm58 is identical with that of pm60 apart from residues 3 (Tyr/Arg) and 6 (Asn/Ser).

Interestingly, photoaffinity-labelling of pm60, but not pm58, was efficiently inhibited by the flavonoid glucoside rutin (3-(6-O-(6-deoxy- α -L-mannopyranosyl)- β -D-glucopyranosyl)oxy}quercetin). We observed that labelling was only partially reduced by the flavonoids quercetin and kaempferol, indicating that the β -glycosidically bound sugar residues of rutin are responsible for the efficient reduction of [^3H]N $_3$ IAA labelling. The different influence of rutin on [^3H]N $_3$ IAA labelling of pm60 and pm58 also hints at a functional difference between pm60 and pm58. Using PNPG as a substrate, preliminary *in vitro* data suggest that p58, too, has β -glucosidase activity (R. Zettl and K. Palme, unpublished work).

The purity of the membrane vesicles used for our solubilization experiments was assessed previously by marker-enzyme analysis (Feldwisch et al., 1992). Analysis of organelle-specific marker enzymes revealed that these vesicles were essentially free of mitochondrial and tonoplast membranes. A 3.4-fold decrease in the endoplasmic-reticulum-specific marker enzyme cytochrome *c* reductase and a 5.3-fold increase in activity of the plasma-membrane-specific vanadate-sensitive ATPase suggested that the vesicles obtained contained more than 90 % plasma membranes. However, at present we cannot exclude the possibility of modest contamination with organelle membranes such as those of plastids.

In fact the degree of chloroplast removal from plasma membranes isolated by aqueous two-phase partitioning is species- and tissue-dependent (Kjellbom and Larsson, 1984). Furthermore, separation of membrane vesicles containing intact chloroplasts (class III) into the poly(ethylene glycol) phase have been described (Larsson et al., 1971). Recently, using immunocytochemistry, we have localized the p60 to proplastids of the coleoptile and the

primary leaf of etiolated maize seedlings (P. Kristoffersen, C. Kruse, B. Brzobohaty, L. Bako, I. Höhfeld, M. Melkonian and K. Palme, unpublished work). Within the proplastids of vascular parenchyma cells of the primary leaf, p60 appears in a paracrystalline structure. This localization of p60 might also explain the different solubilities of p60/p58 and pm60/pm58 in Triton X-100 and Triton X-114 respectively. Soluble and insoluble forms of β -glucosidases in maize have also been found by others (Esen and Cockmus, 1990; Nagahashi et al., 1991).

We were able to demonstrate by immunoprecipitation experiments that pm60 has β -glucosidase activity and contributes to most (i.e. more than 90 %) of the vesicle-associated β -glucosidase activity. The properties of this β -glucosidase activity (pH optimum, K_m and V_{max}) were similar to β -glucosidase activities reported recently by Nagahashi et al. (1991). Those authors showed the presence of cytosolic, cell-wall and particle-associated β -glucosidase activities in maize roots. From these studies it is evident that β -glucosidases localized in the different compartments of maize root cells differ slightly in their kinetic values (with PNPG as a substrate) and in their pH optima.

Both photoaffinity-labelling of pm60 with [^3H]N $_3$ IAA and competitive inhibition of the pm60-specific β -glucosidase activity by 1-NAA and IAA suggest that auxin may play an important role in the control of this enzyme activity. Recently it has been shown, that a β -glucosidase (Zm-p60.1) from maize, which is virtually identical with pm60/p60, cleaved biologically inactive cytokinin *O*-glucosides and kinetin N $_3$ glucoside, releasing active cytokinin (Brzobohaty et al., 1993). Tobacco (*Nicotiana tabacum*) protoplasts that transiently express Zm-p60.1 can use the inactive cytokinin glucosides to initiate cell division. Interestingly IAA glucose ester is a very effective inhibitor of this cytokinin glucoside hydrolysis (B. Brzobohaty, unpublished work). The possible interaction of auxins with the cytokinin conjugate metabolism may provide future insights into the control of plant growth and development by phytohormones.

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REFERENCES

- Bilang, J., Macdonald, H., King, P. J. and Sturm, A. (1993) *Plant Physiol.* **102**, 29–34.
- Blake, M. S., Johnston, K. H., Russel-Jones, G. J. and Gotschlich, E. C. (1984) *Anal. Biochem.* **136**, 175–179.
- Bordier C. (1981) *J. Biol. Chem.* **256**, 1604–1607.
- Brzobohaty, B., Moore, I., Kristoffersen, P., Bako, L., Campos, N., Schell, J. and Palme, K. (1993) *Science* **262**, 1051–1054.
- Campos, N., Feldwisch, J., Zettl, R., Boland, W., Schell, J. and Palme, K. (1991) *Technique* **3**, 69–75.
- Campos, N., Laszlo, B., Feldwisch, J., Schell, J. and Palme, K. (1992) *Plant J.* **1**, 675–684.
- Catty, D. and Raykundalia (1988) in *Antibodies*, vol. 1: Production And Quality Control Of Polyclonal Antibodies (Catty, D., ed.), pp. 19–79, IRL Press, Oxford.
- Esen, A. (1992) *Plant Physiol.* **98**, 174–182.
- Esen, A. and Cocmus, C. (1990) *Biochem. Genet.* **28**, 319–336.
- Estruch, J. J., Chriqui, D., Grossmann, K., Schell, J. and Spena, A. (1991a) *EMBO J.* **10**, 2889–2895.
- Estruch, J. J., Schell, J. and Spena, A. (1991b) *EMBO J.* **10**, 3125–3128.
- Feldwisch, J., Zettl, R., Hesse, F., Schell, J. and Palme, K. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 475–479.
- Gonzalez-Candelas, C., Ramon, D. and Polaina, J. (1990) *Gene* **95**, 31–38.
- Harlow, E. and Lane, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Hicks, G. R., Rayle, D. L., Jones, A. M. and Lomax, T. L. (1989a) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 4948–4952.
- Hicks, G. R., Rayle, D. L. and Lomax, T. L. (1989b) *Science* **245**, 52–54.
- Hösel, W. and Conn, E. E. (1982) *Trends Biochem. Sci.* **6**, 219–221.

- Jacobs, M. and Rubery, P. H. (1988) *Science* **241**, 346–349
- John, M. and Schmidt, J. (1985) *Methods Enzymol.* **160**, 662–671
- Jones, A. M. and Veris, M. A. (1989): *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6153–6156
- Kjellbom, P. and Larsson, C. (1984) *Physiol. Plant.* **62**, 501–509
- Kohchi, C. and Toh-e, A. (1985) *Nucleic Acids Res.* **13**, 6273–6282
- Kosuge, T., Comai, L. and Glass, N. L. (1983) in *Plant Molecular Biology* (Goldberg, R., ed.), Alan R. Liss, New York
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Larsson, C., Collin, C. and Albertsson, P.-Å. (1971) *Biochim. Biophys. Acta* **245**, 425–438
- Liebig, J. and Wöhler, F. (1837) *Annalen* **22**, 11–24
- Love, D. R., Fisher, R. and Bergquist, P. L. (1988) *Mol. Gen. Genet.* **213**, 84–92
- Macdonald, H., Jones, A. M. and King, P. J. (1991) *J. Biol. Chem.* **266**, 7393–7399
- Nagahashi, G., Rao, J., Patterson, D. L. and Tu, S. (1991) *Plant Sci.* **76**, 185–191
- Palme, K. and Schell, J. (1993) *Semin. Cell Biol.* **4**, 81–86
- Skinner, M. K. and Griswold, M. D. (1983) *Biochem. J.* **209**, 281–284
- Zettl, R., Schell, J. and Palme, K. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 689–693