Identification and characterization of plant glycerophosphodiester phosphodiesterase

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GPX-PDE (glycerophosphodiester phosphodiesterase; EC 3.1.4.46) is a relatively poorly characterized enzyme that catalyses the hydrolysis of various glycerophosphodiester (glycerophosphocholine, glycerophosphoethanolamine, glycerophosphoglycerol, glycerophosphoserine and bis-glycerophosphoglycerol), releasing *sn*-glycerol 3-phosphate and the corresponding alcohol. In a previous study, we demonstrated the existence of a novel GPX-PDE in the cell walls and vacuoles of plant cells. Since no GPX-PDE had been identified in any plant organism, the purification of GPX-PDE from carrot cell walls was attempted. After extraction of cell wall proteins from carrot cell suspension cultures with CaCl₂, GPX-PDE was purified up to 2700-fold using, successively, ammonium sulphate precipitation, gel filtration and concanavalin A–Sephadex. Internal sequence analysis of a 55 kDa protein identified in the extract following 2700-fold purification revealed strong similarity to the primary sequence of GLPQ, a bacterial GPX-PDE. To confirm the identity of

plant GPX-PDE, an *Arabidopsis thaliana* cDNA similar to that encoding the bacterial GPX-PDE was cloned and overexpressed in a bacterial expression system, and was used to raise antibodies against the putative *Arabidopsis thaliana* GPX-PDE. Immunochemical assays performed on carrot cell wall proteins extracted by CaCl₂ treatment showed a strong correlation between GPX-PDE activity and detection of the 55 kDa protein, validating the identity of the plant GPX-PDE. Finally, various properties of the purified enzyme were investigated. GPX-PDE is a multimeric enzyme, specific for glycerophosphodiester, exhibiting a *K_m* of 36 μ M for glycerophosphocholine and active within a wide pH range (from 4 to 10). Since these properties are similar to those of GLPQ, the bacterial GPX-PDE, the similarities between plant and bacterial enzymes are also discussed.

Key words: *Arabidopsis*, carrot, cell wall, glycerophosphocholine, glycerophosphodiester phosphodiesterase, plant.

INTRODUCTION

In a previous study [1], NMR and biochemical analysis revealed the existence of a new GPX-PDE (glycerophosphodiester phosphodiesterase; EC 3.1.4.46) activity in various plant cell cultures. This activity was detected using GroPCho (glycerophosphocholine) as the initial substrate, which was hydrolysed into *sn*-glycerol 3-phosphate and choline. The activity was localized on the cell wall and in the vacuole sap of carrot (*Daucus carota*) cells. Competition assays performed on GPX-PDE from carrot cell wall using various phosphodiester showed that GPX-PDE displayed a specificity for glycerophosphodiester [GroPCho, GroPEtn (glycerophosphoethanolamine), GroPIIns (glycerophosphoinositol), GroPGro (glycerophosphoglycerol) and GroPSer (glycerophosphoserine)], but remained unaffected by other phosphodiester (bis-*p*-nitrophenyl phosphate, ADP-ribose and UDP-glucose). These features enabled us to distinguish carrot GPX-PDE from other plant phosphodiesterases described previously [2–5].

At the time our investigations started, GPX-PDE genes and activities had only been characterized in prokaryotic organisms such as *Escherichia coli* [6], *Haemophilus influenzae* [7] and *Borrelia hermsii* [8]. In eukaryotic organisms, GPX-PDE had been reported at the cell surface of *Saccharomyces uvarum* and *Saccharomyces cerevisiae* [9,10], while in animals several GPX-PDEs hydrolysing GroPCho have been observed in liver [11,12],

in kidney cells [13,14], in uterine secretion [15] and in brain [16]. More recently, Mir16, a homologue of a bacterial GPX-PDE, was identified in mammal cells as a protein interacting with RGS16 (regulator of G-protein signalling 16), a factor involved in the regulation of G-protein signalling [17,18]. Overexpression of this Mir16 factor in HEK293T cells showed that it selectively hydrolyses GroPIIns, suggesting that it is involved in phosphoinositide metabolism.

The absence of data concerning the molecular characterization of plant GPX-PDEs encouraged us to carry out GPX-PDE purification from the carrot cell wall. This approach revealed the existence of a 55 kDa protein similar to GLPQ, a bacterial GPX-PDE characterized previously in *E. coli*. Isolation of an *Arabidopsis thaliana* cDNA encoding a putative GPX-PDE allowed the production of antibodies that specifically recognized the GPX-PDE isolated from carrot cell wall. The enzymic properties of carrot GPX-PDE were compared with results described previously at the cellular level.

EXPERIMENTAL

Plant material

Carrot (*Daucus carota*) cell suspension cultures were grown in Murashige and Skoog liquid medium (Sigma) supplemented with

Abbreviations used: EST, expressed sequence tag; GPX-PDE, glycerophosphodiester phosphodiesterase; GroPCho, glycerophosphocholine; GroPEtn, glycerophosphoethanolamine; GroPIIns, glycerophosphoinositol; GroPGro, glycerophosphoglycerol; GroPSer, glycerophosphoserine; ORF, open reading frame; RACE, rapid amplification of cDNA ends; RGS, regulator of G-protein signalling.

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30 g · litre⁻¹ sucrose and 0.1 mg · litre⁻¹ 2,4-dichlorophenoxy-acetic acid. *Arabidopsis thaliana* cell suspension cultures were grown in Gamborg B5 medium (Sigma) supplemented with 15 g · litre⁻¹ sucrose and 0.1 mg · litre⁻¹ 2,4-dichlorophenoxy-acetic acid.

Chemicals

GroPCho was purchased from Sigma. Cadmium was removed by elution through an IRC-50 cation exchange column equilibrated with 100 mM potassium acetate, pH 6. [³H]GroPCho was prepared from [³H]phosphatidylcholine by mild deacylation [1].

Protein extraction and purification

Cell wall proteins were first extracted from 4-day-old carrot cell suspension cultures treated with CaCl₂ using a protocol adapted from [19]. Intact cells were harvested by filtration and rinsed carefully with water. Then the cells were stirred in 5 vol. (volume/fresh weight) of 0.2 M CaCl₂/20 mM Tris, pH 7.4, for 5 min at 30 °C. After removing the cells by filtration, the saline extract was dialysed against 20 mM Tris, pH 7.4, and concentrated by ultrafiltration on a PM-30 membrane (Amicon) until the protein concentration reached 0.5 mg · ml⁻¹. (NH₄)₂SO₄ was added to reach 40 % saturation. After centrifugation (20 000 g, 15 min), the pellet was discarded. The proteins were precipitated by addition of (NH₄)₂SO₄ to 60 % saturation. After centrifugation (20 000 g, 15 min), the pellet was resuspended in 20 mM KH₂PO₄/K₂HPO₄, pH 6.9, and desalted by elution through a PD-10 column (Pharmacia) in the same buffer. The extract was then subjected to gel filtration chromatography on a Superdex 200 column pre-equilibrated in 20 mM KH₂PO₄/K₂HPO₄ and 150 mM KCl, pH 6.9. Elution was carried out with the same buffer. Fractions exhibiting high GPX-PDE activity were loaded on to a concanavalin A–Sephacrose column equilibrated in 40 mM Tris, 1 mM MgCl₂ and 1 mM CaCl₂, pH 7.4. The column was first washed with the equilibration buffer and then rinsed with 0.5 M KCl in the same buffer. Proteins were eluted after addition of 2 % (w/v) methyl mannose to the rinsing buffer.

The native molecular mass of GPX-PDE was determined, after gel filtration, from a plot of *K*_{av} (partition coefficient) against log (molecular mass) of protein standards. Protein content was measured using the colorimetric assay of either Lowry et al. [20] (for high concentrations) or Bradford [21].

Enzymic assays

GPX-PDE activity was measured using a two-step enzymic assay as described previously [1]. At high concentrations of substrate (> 0.5 mM), GroPCho (or GroPEtn, GroPIns, GroPGro or GroPSer) was first hydrolysed in 50 mM Mes and 5 mM CaCl₂, pH 6, at 30 °C. The reaction was stopped by the addition of perchloric acid [up to 3 % (w/v) final]. In the second step, the choline or the glycerol and the glycerol 3-phosphate released were measured by enzymic analysis [1]. At lower concentrations of GroPCho, enzymic assays were performed by incubating [³H]GroPCho (1.5 µCi/µmol) in a reaction buffer comprising 50 mM Mes and 5 mM CaCl₂, pH 6. At different times (from 0 to 60 min), a fraction (100 µl) was collected and the reaction was stopped by heating for 10 min at 95 °C in a water bath. To prevent potential errors induced by heating, the test was only performed using low enzymic activities (usually from 2 to 20 nmol · h⁻¹ · ml of reaction mixture⁻¹); choline was therefore released at a constant rate. After drying, the mixture was resuspended in ethanol/water (50:50, v/v) and loaded on to a silica-gel plate. [³H]Choline

and [³H]GroPCho were separated by TLC in a methanol/0.5 % NaCl/NH₄OH (100:100:2, by vol.) running buffer, as described in [22].

Cloning of an *Arabidopsis* cDNA encoding a GPX-PDE-like protein

Purification of total RNA from *A. thaliana* roots, shoots and leaves was performed using the Rneasy Plant Mini Kit from Qiagen Laboratories. Purification of mRNA from the total RNA fraction was performed using the mRNA Direct Kit from Dynal Laboratories. Construction of an *A. thaliana* cDNA library was performed using the Marathon cDNA Amplification Kit from Clontech Laboratories. All RACE (rapid amplification of cDNA ends)/PCR experiments were performed using the Advantage cDNA Polymerase Mix (Hot-start and proofreading enzyme) from Clontech Laboratories.

Positive amplification was obtained from RACE/PCR performed with the forward primer 5'-PDE (AACTGACAGAGTGGGATAAGAGAC) in combination with the Adaptor Primer 1 (CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT) provided with the Marathon cDNA Amplification Kit from Clontech Laboratories. Clones were inserted into the pBluescript KS vector and sequenced from both ends with standard T3 and T7 primers as well as with insert-specific primers.

Overexpression of the putative *Arabidopsis* GPX-PDE and synthesis of polyclonal antibodies

A truncated cDNA of the putative *Arabidopsis* GPX-PDE flanked by *Asn*I and *Bam*HI restriction sites on its 5' and 3' extremities respectively was amplified using the forward primer 5'-Asn (GATCATTAATGGGAGGACATTGCACCCTCTCCCTG) and the reverse primer 3'-Bam (GATCGGATTCTCAAGCCTTTGCAT-AAGGAG), and was inserted into the pBluescript KS vector. The *Asn*I–*Bam*HI fragment was then excised and cloned in the *Nde*I and *Bam*HI restriction sites of the pET15b (Novagen) expression vector. Overexpression was achieved in the *E. coli* BLR(DE3) strain (Novagen) after induction for 3 h at 37 °C with 0.1 mM isopropyl β-D-thiogalactoside. After cell lysis by sonication, the inclusion bodies were isolated by centrifugation. In order to remove membrane contaminants, the insoluble pellet was treated with 1 % (w/v) Triton X-100. Solubilization of inclusion bodies was achieved in 6 M urea. The recombinant protein was purified by nickel affinity chromatography according to the instructions from the manufacturer (Novagen). Rabbit polyclonal antibodies were raised against the overexpressed GPX-PDE-like protein at the Elevage Scientifique des Dombes (F-01400, Châtillon sur Chalaronne, France).

Western blot analyses were performed after SDS/PAGE separation. Due to the high specificity of the antibodies, it was also possible to detect the protein using dot-blot analyses: a small volume (2.5 µl) of the protein extract was loaded directly on to a nitrocellulose membrane and dried for 30 min. Putative GPX-PDE was detected with the immune serum at a 1:5000 dilution using peroxidase and enhanced chemiluminescence detection. No signal was observed with pre-immune serum (results not shown).

MS and protein identification

After separation by SDS/PAGE and Coomassie Blue staining, the 55 kDa protein band was excised from the gel and subjected to trypsin digestion. Proteins were extracted from the gel with 5 % (v/v) formic acid and then with acetonitrile. The extracts were combined with the original digest and the sample was

Table 1 Purification of GPX-PDE from carrot cell suspension cultures

GPX-PDE activity was measured as the quantity of GroPCho hydrolysed per h per mg of protein, as described in the Experimental section.

Purification step	Protein (mg)	Specific activity ($\mu\text{mol} \cdot \text{h}^{-1} \cdot \text{mg}^{-1}$)	Purification (fold)	Yield (%)
Carrot cells (200 g)	4000	0.24	—	100
CaCl ₂ cell wall extract	67	2.3	9.5	16
Ammonium sulphate precipitation	17	4.4	18	7.8
Superdex-200	0.4	120	500	6.2
Concanavalin A–Sephadex	0.03	650	2700	2.0

evaporated to dryness in a vacuum centrifuge. The residues were dissolved in 0.1 % (v/v) formic acid and desalted using a Zip Tip (Millipore). Elution of the peptides was performed with 5–10 μl of a 50:50:0.1 (by vol.) acetonitrile/water/formic acid solution. The peptide solution was introduced into a glass capillary (Protana) for nanoelectrospray ionization.

Tandem MS experiments were carried out on a Q-TOF hybrid mass spectrometer (Micromass) in order to obtain sequence information. Collision-induced dissociation of selected precursor ions was performed using argon as the collision gas and with collision energies of 40–60 eV. MS/MS sequence information was used for database searching using the following programs: (a) MS-Edman from the University of California San Francisco (<http://prospector.ucsf.edu/>), (b) BLAST at the NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>) and (c) FASTS or TFASTS from the University of Virginia (<http://fasta.bioch.virginia.edu/fasta/cgi/>). Predictions of subcellular location and maturation site were achieved using the software P-SORT (<http://psort.nibb.ac.jp/>) [23], TargetP and SignalP (<http://www.cbs.dtu.dk/services/>) [24,25].

MS/MS spectra analyses

Interpretation of MS/MS spectra was achieved manually and with the help of the MaxEnt3 program (MassLynx software; Micromass). Calculation of mass differences between consecutive fragment ions, generated by collision-induced dissociation, provided sequence information. The data obtained by MS/MS analyses for the 55 kDa protein band and corresponding to the putative GPX-PDE are given in Table 2. These sequences were used for the database research.

RESULTS

Partial purification of GPX-PDE from carrot cell wall

Treatment of intact carrot cells with 0.2 M CaCl₂ was a simple way to extract the GPX-PDE from the cell wall. As shown previously using enzymic markers for intracellular compartments [1], this extraction released mainly extracellular proteins. Using these conditions, the specific activity of GPX-PDE was enriched 9.5-fold (Table 1). It was therefore decided to continue GPX-PDE purification from this point. A purification procedure with three more steps was developed, as recapitulated in Table 1. In the first step, proteins were precipitated with 60 % saturation ammonium sulphate after discarding the 40 % saturation precipitate. Secondly, separation by gel filtration using a Superdex-200 FPLC column was performed. Lastly, since many cell wall proteins are glycosylated, separation was performed on a concanavalin A–Sephadex column, and GPX-PDE was eluted

Figure 1 Analysis by SDS/PAGE (A) and Western blot (B) of the products of GPX-PDE purification from carrot cells

Lane 1, crude cellular extract; lane 2, cell wall protein extracted after CaCl₂ treatment; lane 3, cell wall protein after ammonium sulphate precipitation; lane 4, protein extract after gel filtration on Superdex-200; lane 5, protein extract after elution on concanavalin A–Sephadex; lane M, molecular mass markers (kDa).

Table 2 Interpretation of MS/MS spectra: peptide sequences obtained from the 55 kDa protein

The four peptide sequences were obtained from the 55 kDa protein fraction obtained by SDS/PAGE (see Figure 1). Some mass differences are isobaric and thus correspond to several different amino acids. Leucine and isoleucine are isobaric (denoted as [IL]). The presence of K or R (denoted [KR]) was assumed because peptides were recovered from trypsin digestion

Peptide mass	Peptide sequence
1098.4	R*P[IL]YP[IL]PSR
1161.5	[KR]NPV[IL][IL]NQHVK
1711.8	[KR]GSNGE[IL]PEETAAAYMR
1851.8	[KR]A[IL]EEGDF[IL]ETD[IL][IL]ASK

* In contrast with the other peptide sequences, the initial arginine belongs to the 1098.4 peptide.

with 0.5 M KCl/2 % (w/v) α -methylmannose. After these steps, GPX-PDE was purified up to 2700-fold (Table 1). Addition of a further step (e.g. separation on a DEAE-trisacryl or a MonoQ column) hardly enriched the protein, but resulted in an extensive loss of activity. Due to this low recovery (Table 1), the purification was stopped after the concanavalin A–Sephadex column. Electrophoretic analysis on SDS/PAGE of the 2700-fold-purified fraction showed two major bands at 55 and 35 kDa and three minor bands at 75, 21 and 15 kDa (Figure 1A).

Similarity between the 55 kDa protein and a bacterial GPX-PDE

The 55 kDa band obtained after protein purification was excised from the SDS/PAGE gel and subjected to peptide sequencing. Digestion with trypsin and sequencing of inner fragments revealed four peptides (Table 2) with similarity to two *A. thaliana* ORFs (open reading frames) annotated as a putative GPX-PDE (accession nos. NP_177561 and NP_196420). The comparison between the carrot cell wall GPX-PDE internal peptide sequences and the two *Arabidopsis* putative GPX-PDE sequences (Figure 2) showed a high degree of similarity (96 % identity or similarity). The *Arabidopsis* ORF annotation was based on similarity to GLPQ, a bacterial GPX-PDE characterized in *E. coli* [6], and also in *Haemophilus influenzae* [7] and *Borrelia hermsii* [8]. Indeed, the comparison of *Arabidopsis* GPX-PDE and *E. coli* GLPQ sequences shows significant similarity (55 % identity or similarity). Moreover, from the sequence alignment, it can be seen that some domains are highly conserved between species, suggesting that they may contain critical residues essential for enzymic activity (Figure 3A).

Figure 2 Alignment of the carrot cell wall 55 kDa protein inner fragments with *Arabidopsis thaliana* ORFs and a *Lycopersicon esculentum* EST annotated as putative GPX-PDEs

(A) Alignment of three fragments in the N-terminal region; (B) alignment of the final carrot fragment in the core of the putative protein. The arrow indicates the predicted cleavage signal. Accession numbers of the sequences used in the analysis are as follows: A. thal. I, NP_177561; A. thal. V, NP_196420; Lyc. esc., A1895134, AW649499 and AW650096. Similarity between residues was defined by the belonging to the following groups: ASPTG, ILMV, KRH, NQ, DE, FWY, C.

Figure 3 Relationship between *A. thaliana* putative GPX-PDE and various members of the GPX-PDE superfamily

(A) Alignment between conserved regions of different GPX-PDEs. Invariant residues are shaded in black; similar residues are in grey. Numbers indicate the positions of amino acids. Accession numbers of the sequences used in the analysis are as follows: A. thal. I, NP_177561; A. thal. V, NP_196420; *Oryza sativa*, AP003274; *E. coli* glpQ, X56907; *H. sapiens* Mir16, NP_057725; *E. coli* ugpQ, X14437; *S. cerevisiae* YPL206c; AtGPIP2 (*Arabidopsis* glycosylphosphatidylinositol-anchored arabinogalactan protein), AAL07129.1. (B) Phylogenetic analysis of the GPX-PDE family. The analysis was performed using all sequences listed in the Interpro database (<http://www.ebi.ac.uk/interpro>); the phylogenetic tree was after restricted to species of interest. Abbreviations for species: *H.s.*, *Homo sapiens*; *E.c.*, *Escherichia coli*; *B.s.*, *Bacillus subtilis*; *S.c.*, *Saccharomyces cerevisiae*; *A.t.*, *Arabidopsis thaliana*; *O.s.*, *Oryza sativa*; *T.e.*, *Triticum aestivum*; *Ana.*, *Anabaena*; *R.s.*, *Ralstonia solanaceum*; *B.h.*, *Borrelia hermsii*.

Cloning of the cDNA encoding the putative GPX-PDE from *A. thaliana*

To confirm the identification of plant GPX-PDE, we isolated an *Arabidopsis thaliana* cDNA encoding the GPX-PDE-like protein. The cDNA isolated corresponded to a gene located on chromosome I (accession no. NP_177561 [26]). The full-length cDNA codes for a protein of 392 residues with a calculated molecular mass of 45 kDa. The complete sequence of the cDNA was in agreement with the intron/exon prediction. Based on signal prediction programs, the hydrophobic N-terminal region was predicted to be a peptide signal, with a cleavage signal predicted between residues 21 and 22: (Ala-Gly↓Arg-Thr-Leu; see Figure 2). Four N-glycosylation sites (-Asn-Xaa-Ser/Thr-) were also predicted. Analysis of the *Arabidopsis* genome revealed the existence of a second homologue of the bacterial GPX-PDE, located on chromosome V (accession no. NP_196420). This second gene codes for a protein of 372 residues with a calculated molecular mass of 42.8 kDa. A peptide signal with a cleavage site in the N-terminal region and three putative N-glycosylation sites were predicted.

Immunodetection of GPX-PDE activity using antibodies raised against putative *A. thaliana* GPX-PDE

In order to overexpress the recombinant putative *A. thaliana* GPX-PDE in *E. coli*, the cDNA encoding the 392-amino-acid protein was cloned into the pET-15b plasmid. The cDNA insert was truncated at the N-terminal extremity in order to remove the putative peptide signal. Expression in the BLR(DE3) strains led to massive production of insoluble protein that accumulated as inclusion bodies. Since all attempts to solubilize the recombinant protein failed, the overexpression products were only used to raise antibodies against putative *Arabidopsis* GPX-PDE purified from inclusion bodies.

Antibodies were tested on the recombinant protein and on different extracts from carrot and *Arabidopsis* cells. Interestingly, Western-blot analyses performed on cell wall proteins extracted from carrot cell cultures showed that the antibodies cross-reacted with a large band corresponding to a molecular mass ranging from 54 to 57 kDa (Figure 1B). Moreover, this large band was still detected during the subsequent purification steps (Figure 1B). To confirm further the correlation between GPX-PDE enrichment and detection with the antibodies, we compared, using dot-blot analyses, the GPX-PDE activity and the immunochemical signal during all purification steps (Figure 4). Remarkably, the cross-binding intensity reflected the GPX-PDE activity measured in the different fractions eluted from the Superdex-200 (Figure 4A) and the concanavalin A-Sepharose (Figure 4B) columns, thus indicating that the immunochemical signal was specifically associated with purified fractions exhibiting a strong GPX-PDE activity.

Immunodetection of GPX-PDE in the vacuoles of carrot and *Arabidopsis*

The existence of two distinct genes encoding putative GPX-PDEs in *A. thaliana* also raises the question of the location of the different GPX-PDEs. In the present work, we focused on carrot cell wall GPX-PDE; however, GPX-PDE activities have also been reported in two compartments in carrot and *A. thaliana*: cell wall and vacuole sap [1]. Preliminary experiments performed on carrot and *Arabidopsis* vacuoles isolated by flotation showed that the antibodies raised against the GPX-PDE-like protein cross-hybridized with a single protein of 54–55 kDa (results not shown), suggesting similarities between cell wall and vacuolar

Figure 4 Correlation between GPX-PDE activity and hybridization with antibodies directed against a putative GPX-PDE during enzyme purification from carrot cell wall

GPX-PDE activity was measured and immunochemical signals were detected in the different fractions eluted from the Superdex-200 (A) or the concanavalin A-Sepharose (B) columns. The immunochemical signal was measured by dot-blot. '+ KCl' and '+ man' correspond to the addition of 0.5 M KCl and 0.5 M α -methylmannopyranoside respectively in the concanavalin A elution medium.

GPX-PDEs. However, it is not clear whether the location of the enzyme in the vacuole results from the existence of two different genes or from different post-translational modifications or targeting.

Properties of the cell wall GPX-PDE

Structural properties

Purification of GPX-PDE provided information about some of its structural properties. First, after gel filtration on the Superdex-200 column, the native molecular mass was estimated to be 280 kDa by comparison with the K_{av} (partition coefficient) of protein standards, thus indicating that GPX-PDE is a multimeric enzyme. Secondly, retention of GPX-PDE on the concanavalin A-Sepharose column suggested that it is a glycoprotein. Since glycoproteins are known to bind poorly to SDS [5,27], we tested GPX-PDE activity in the presence of SDS. Remarkably, GPX-PDE remained active in the presence of 2% (w/v) SDS. Protein denaturation could only be achieved by heating. Finally, the presence of glycosylated residues is consistent with a monomer molecular mass (55 kDa) higher than the molecular mass predicted by the different cDNA clones encoding putative GPX-PDE (from 43 to 45 kDa).

Enzymic properties

Various enzymic assays were performed on the 2700-fold-purified GPX-PDE. In order to test the influence of pH on GPX-PDE activity, we measured the rate of GroPCho hydrolysis in different buffers. Surprisingly, GPX-PDE remained active over a large pH range (from 4 to 10), showing an optimum at pH 9.5. This limited influence of pH is consistent with previous NMR studies, which revealed activity of extracellular GPX-PDE at both acidic and alkaline pH values [1], as observed for other hydrolases [28]. The K_m for GroPCho was estimated at 36 μ M, in good agreement with the results obtained previously with carrot cell wall protein extract [1]. The V_{max} for GroPCho was 10.8 μ mol $\cdot$ min $^{-1}$ $\cdot$ mg of protein $^{-1}$ (μ kat) (Table 3).

Since phosphodiesterases characterized previously were found to be affected by bivalent metal ions [3,29], we tested the effects

Table 3 V_{max} of GPX-PDE measured for different glycerophosphodiesterases

Activities were measured using the 2700-fold-purified GPX-PDE from carrot. Activities are expressed as μ mol of GPX hydrolysed per min per mg of protein (μ kat).

Substrate	V_{max} (μ mol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$)
GroPCho	10.8
GroPEtn	8.6
GroPIns	7.7
GroPGro	8.8
GroPSer	8.5

of EDTA and of different ions (Ca^{2+} , Mg^{2+} , Mn^{2+} , Co^{2+} , Zn^{2+} , Cu^{2+} and Fe^{2+}). At high concentrations (5 mM), Zn^{2+} , Cu^{2+} or EDTA caused a minor inhibition (40%), whereas Fe^{2+} had a much stronger effect, since the GPX-PDE activity was strongly inhibited (> 90%) at concentrations exceeding 100 μ M (results not shown).

We also tested the substrate specificity of the 2700-fold-purified enzyme. Interestingly, the same extract hydrolysed GroPEtn, GroPIns, GroPGro, GroPSer and GroPGro at comparable rates (Table 3), while no hydrolytic activity was detected using other phosphodiesterases such as bis-*p*-nitrophenyl phosphate, ADP, ADP-ribose, ADP-glucose and UDP-glucose. Overall, these results confirm the competition assays performed on the cell wall extracts [1], which suggested that plant GPX-PDE was specific for glycerophosphodiesterases, reflecting the properties of the *E. coli* GLPQ enzyme [29,30].

GPX-PDE belongs to a highly conserved family

The strong similarity between the internal peptide of the carrot GPX-PDE and both putative *A. thaliana* GPX-PDEs enabled us to identify the carrot 55 kDa protein. When the analysis was extended to genomic or EST (expressed sequence tag) sequences from other flowering plants (*Lycopersicon esculentum*, *Solanum tuberosum*, *Zea mays*, *Medicago truncatula*, *Hordeum vulgare* and *Oryza sativa*), it revealed the existence of a highly conserved family among plant species. Indeed, the similarity varied from 83% (*H. vulgare*) to 90% (*M. truncatula*). The variability was much higher in the N-terminal region, where the hydrophobic peptide signals are predicted (results not shown).

In addition to these strong similarities, the plant GPX-PDE described in the present work also shares identity with a wider class of proteins listed in the InterPro database (<http://www.ebi.ac.uk>) as proteins that contain a glycerophosphoryl diester phosphodiesterase domain. In this family are found GLPQ and UGPQ, the two GPX-PDEs described previously in *E. coli* [6] and in other prokaryotic organisms. The family also contains mammalian Mir16 factor, a GroPIns phosphodiesterase identified for its ability to bind to RGS16, involved in the regulation of G-protein signalling [17,18]; the Mir16 factor is more similar to the UGPQ phosphodiesterase, while plant GPX-PDEs are more similar to GLPQ (Figures 3A and 3B). Finally, the *Arabidopsis* genome sequence revealed the existence of two sets of proteins distantly related to plant GPX-PDE (Figure 3B): the first set consists of seven arabinogalactan proteins identified recently as glycosylphosphatidylinositol-anchored proteins (accession nos. A.t. BAA96908.1, BAB10996.1, CAB36515.1, AAL07129.1, AAF98209.1, BAB08565.1 and AAF98210.1; [31,32]), while the second set comprises four unknown proteins (BAB09710.1, AAF14831.1, BAB10593.1 and AAL32735.1). However, the enzymic activities of these plant homologues have not yet been

Table 4 Comparison of the properties of carrot GPX-PDE and bacterial GLPQ GPX-PDE

The properties of GLPQ were investigated for the enzymes from *E. coli* [29,30] and *Borrelia hermsii* [33].

Properties	Carrot GPX-PDE	Bacterial GPX-PDE (GLPQ)
Structural properties		
Native mass (kDa)	275	70–75
Denatured mass (kDa)	55	38
Primary sequence length (aa)	372–392	357
Predicted molecular mass (kDa)	43–45	41
Cleavage site	N-terminal signal peptide; putative cleavage site at aa 21	N-terminal extremity, cleavage at aa 25
Location	Cell wall, vacuole	Periplasm
Enzymic properties		
K_m (μ M) (for GroPCho)	36	280
V_{max} (μ kat)	10.8 (purification 2700-fold)	170
Substrate specificity	Glycerophosphodiesterases	Glycerophosphodiesterases
Optimal pH	9.5 (but little influence of pH on enzyme activity)	7.8 (but little influence of pH on enzyme activity)
Inhibitors	Weak inhibition by EDTA, Mn^{2+} , Co^{2+} , Cu^{2+} , Zn^{2+} ; strong inhibition by Fe^{2+}	Strong inhibition by EDTA; activation by Ca^{2+}
Possible regulation of expression	Activation by phosphate deprivation	In <i>E. coli</i> , modulation by carbon supply; in <i>B. hermsii</i> , activation by phosphate deprivation

characterized. The phylogenetic relationship between these sequences is recapitulated in Figure 3(B).

DISCUSSION

The purification of a GPX-PDE from carrot cell wall is, to our knowledge, the first attempt to purify this enzyme from a plant species. Since the amount of the enzyme was extremely low, the purification was stopped at a 2700-fold enrichment factor. This purification factor was sufficient to identify a 55 kDa protein presenting structural similarity to bacterial GPX-PDE. Two experiments confirmed the enzymic function of this 55 kDa protein. First, an antibody raised against an *Arabidopsis thaliana* GPX-PDE-like protein specifically recognized the 55 kDa protein during each step of the purification (Figure 4). Secondly, there was a high correlation between the GPX-PDE activity measured after every purification step and the immunochemical signal detected in these protein extracts. Since the 2700-fold-purified extract contained very few proteins (see Figure 1), it seems reasonable to assume that the 55 kDa protein is indeed responsible for the GPX-PDE activity detected in carrot cell wall.

Furthermore, the striking similarities between *E. coli* GLPQ GPX-PDE and carrot GPX-PDE strongly support the proposal that the carrot 55 kDa protein extracted from carrot cell walls is a plant GPX-PDE. On the one hand, the analysis of the internal peptides of the 55 kDa protein demonstrates strong sequence similarity to the GLPQ protein. In addition, the putative *Arabidopsis* GPX-PDE and *E. coli* GLPQ sequences have similar lengths (between 350 and 392 residues), and both contain a putative N-terminal signal peptide. Furthermore, many enzymic properties exhibited by carrot cell wall GPX-PDE are similar to those of *E. coli* GLPQ, as recapitulated in Table 4. Indeed, both enzymes break down glycerophosphodiesterases, releasing glycerol 3-phosphate and a free alcohol (choline, ethanolamine, glycerol or serine). Bacterial and plant GPX-PDEs share the same substrate specificity, since they both hydrolyse the different glycerophosphodiesterases, but not other phosphodiesterases such as bis-*p*-nitrophenyl phosphate. Both activities are affected by bivalent ions and are able to work at alkaline pH. Lastly, there is a difference regarding the affinity of GPX-PDE for phosphodiesterases, since carrot GPX-PDE exhibited a lower K_m for GroPCho (36 μ M) than the *E. coli* GPX-PDE (280 μ M; see [29,30]).

The existence of a large family of proteins sharing a GPX-PDE domain among eukaryotes and prokaryotes also raises several questions. Since the complete *Arabidopsis* genome contains 13 ORFs encoding putative proteins with a phosphodiesterase domain, the relative importance of these different phosphodiesterases can be questioned. Indeed, the $CaCl_2$ extraction performed in the present study did not release the total GPX-PDE activity measured in intact cells (Table 1). Thus the possibility cannot be ruled out that some extracellular proteins, more particularly the glycosylphosphatidylinositol-anchored arabinogalactan proteins located on the plasma membrane [31,32], participate in the total extracellular GPX-PDE activity measured in our previous paper [1].

In conclusion, the physiological significance of plant GPX-PDEs is not completely understood. Since plant extracellular GPX-PDE activity was enhanced by phosphate deprivation [1], we suggested previously that extracellular GPX-PDE could be involved in rescuing phosphate either from the soil, where the phospholipid content may represent up to 7% of the immobilized phosphate [33], or from phospholipid catabolism or turnover. If this hypothesis was verified, the function of plant extracellular GPX-PDE would present striking analogies with bacterial GLPQ. Indeed, in addition to sequence similarity, comparable enzymic features and location in an extracellular compartment, several bacterial GPX-PDEs are also under metabolic control. In *E. coli*, the *glpQ* gene belongs to the *glp* regulon, which is regulated by carbon availability [30], while in *Borrelia hermsii*, *glpQ* expression is enhanced when the phosphate supply is low [34]. To further challenge this hypothesis and to explore other physiological functions, the use of insertional mutants in the two GPX-PDE genes of *Arabidopsis thaliana* described above or extinction of their gene expression by RNA interference may prove very useful. Such mutants or transformants should then be analysed for their ability to grow on limited amounts of phosphate or with phosphate supplied only in organic form. Phosphorus and phospholipid metabolism in these plants should also be examined carefully.

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Received 30 September 2003/22 December 2003; accepted 29 January 2004

Published as BJ Immediate Publication 29 January 2004, DOI 10.1042/BJ20031489