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Microcystin affinity purification of plant protein phosphatases: PP1C, PP5 and a regulatory A-subunit of PP2A

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Abstract Proteins of ~35, 55 and 65 kDa were purified from cauliflower extracts by microcystin-Sepharose chromatography and identified by amino acid sequencing as plant forms of protein (serine/threonine) phosphatase 1 (PP1) catalytic subunit, PP5 and a regulatory A-subunit of PP2A, respectively. Peptides that corresponded both to the tetratricopeptide (TPR) repeat and catalytic domains of PP5 were identified. Similar to mammalian PP5, the casein phosphatase activity of plant PP5 was activated >10-fold by arachidonic acid, with half-maximal stimulation occurring at ~100 μM lipid.

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Key words: Arachidonic acid; Protein phosphatase 5; Tetratricopeptide repeat; Plant

1. Introduction

The protein phosphatases (PPs) that dephosphorylate serine, threonine and (at least in vitro) histidine residues are encoded by the PPP and PPM gene families that are defined by their distinct amino acid sequences, tertiary structures and sensitivities to inhibitors. The PPP enzymes play critical roles in determining the level of phosphorylation and, hence, the biological activities of many proteins in eukaryotic cells, which may explain why their structures have been so highly conserved during evolution. A phylogenetic tree depicting sequence relationships among the PPP catalytic subunits from humans, *Drosophila melanogaster* and *Saccharomyces cerevisiae* reveals that across these species, the PPP gene family falls into four distinct subfamilies, termed PPP1, PPP2A, PPP2B and PPP5 [1,2].

The catalytic subunits of PP1 and PP2A do not exist as the free catalytic subunits, but are complexed to regulatory subunits. The diverse functions of mammalian PP1 are specified by interactions with different PP1 regulatory subunits which target the catalytic subunits to cellular structures, such as glycogen particles, myosin fibres and spliceosomes, direct the substrate specificity towards colocalised substrates and mediate the regulation of PP1 activity by extracellular stimuli [3,4]. Most known forms of PP2A are complexed to the A-subunit, which in turn interacts with many cellular proteins (B-subunits) that differentially modulate the substrate specificity of PP2A [5]. The catalytic subunits of PP2A and PP4 (in the PPP2A subfamily and originally termed PPX) can also interact with Tap42 and α4, components of the rapamycin-sensitive nutrient sensing pathways in yeast and mammalian cells, respectively [6,7].

The PPP family is the target for a number of drugs, toxins and tumour promoters. For example, members of the PPP1, PPP2A and PPP5 subfamilies are also inhibited by several naturally occurring tumour-promoting toxins, including okadaic acid, the substance responsible for diarrhoetic shellfish poisoning, and microcystins (MCs), the cyclic heptapeptides produced by blue-green algae which are hepatotoxins and a potential threat to water supplies [8].

Members of the PPP1 and PPP2A subfamilies have been identified in plants by their biochemical characteristics, sequences of cDNA and PCR clones and as phenotypic mutants (reviewed in [9]). The profound effects of okadaic acid and MC on plants have implicated the PPP1 and PPP2A subfamilies as regulators of many cellular processes, including defence responses against fungal pathogens and wounding, control of cytosolic enzymes, ion channels, protein turnover, gene expression, sister chromatid separation in meiosis and development of pollen tubes and root hairs (for example, [10–12]). The *Arabidopsis* genome encodes at least two isoforms of PP4, eight isoforms of PP1 [9], at least four PP2A catalytic subunits [13] and regulatory A- and B-subunits of PP2A [14–16]. Disruption of a gene encoding a regulatory A-subunit of PP2A (*RCN1*) has been found to be the genetic basis of an *Arabidopsis* T-DNA insertion mutant that was isolated by its altered root curling and hypocotyl elongation responses to auxin transport inhibitors [17].

Despite being repeatedly implicated as central regulatory components in plants, no plant PPs have been purified to homogeneity [18]. Until recently, the analysis of PPs has been very difficult because of their low concentrations in cells, the complexity of the multiple molecular forms, the susceptibility of regulatory subunits to proteolysis and their tendency to dissociate from the catalytic subunits during purification. However, we have greatly facilitated the analysis of these enzymes by designing a MC-Sepharose column, which proved to be useful for purifying distinct forms of mammalian PP1 [19–21], and here describe the MC affinity purification of plant PPs, including the first identification of a plant lipid-stimulated PP5.

2. Materials and methods

2.1. Materials

MC-LR was from Dr. Linda Lawton (Robert Gordon's University, UK). ^{125}I-labelled MC-YR and MC-Sepharose were prepared as in [19]. Protein assay reagent was from Pierce-Warriner (Chester, UK). Bovine serum albumin (BSA) was used as standard. Miracloth was from Calbiochem. Lipids were dissolved at 60 mM in dimethylsulfoxide, except for arachidic acid which was dissolved at 60 mM in chloroform.

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2.2. Preparation of ^{32}P-labelled substrates and PP assays

^{32}P-labelled phosphorylase *a* (containing 1 mol phosphate per mol subunit) was prepared by phosphorylation with phosphorylase kinase and ^{32}P-labelled casein by phosphorylation with protein kinase A. The dephosphorylation of phosphorylase *a* (10 μM) and casein (6 μM) was assayed by standard procedures [22], but in the absence of BSA and Brij-35, which reduce the effectiveness of fatty acid in activating mammalian PP5 [23]. One unit of activity (U) was the amount of enzyme which catalysed the release of 1 μmol of phosphate from each substrate in 1 min [22].

2.3. Purification of PPs from cauliflower by MC-Sepharose chromatography

The outer curd of one cauliflower (~500 g, *Brassica oleracea* var. *botrytis*, Tesco Supermarket, Dundee, UK) was homogenised in a Waring blender in two volumes ice-cold buffer A (50 mM triethanolamine-HCl, pH 7.5, 0.1 mM EGTA, 5% (v/v) glycerol, 0.1% (v/v) 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine and clarified by centrifugation at 5000×*g* for 20 min to give an extract containing ~200 mg protein. After filtration through glass wool and two layers of miracloth, the sample was made fractionated from 0 to 17% poly(ethylene glycol) (PEG) by addition of a 50% (w/v) solution of PEG 6000 (BDH) in buffer A. The sample was stirred gently for 30 min before centrifugation at 5000×*g* for 20 min. The 0–17% PEG pellet was resuspended to a total volume of 200 ml in buffer A, clarified by centrifugation and mixed end over end for 1 h at 4°C with 1 ml MC-Sepharose (containing 0.17 mg/ml MC-LR). The mixture was poured into a column, washed with buffer A plus 500 mM NaCl (100 ml) and then buffer A containing 2 M KCl until the eluate was less than 0.005 mg/ml in protein. Buffer A containing 3 M NaSCN (0.75 ml, one void volume) was passed through the column and the flow stopped for 30 min. The flow was then re-started and the eluate-containing protein (20–40 μg, spread out over ~100 ml) was collected and concentrated by centrifugation, first in Centriprep-10 and then in Centricon-10 concentrators (Amicon).

For anion-exchange chromatography, the resuspended 0–17% PEG fraction from extract of one cauliflower was applied to a 5×30 cm column of DEAE-Sepharose CL-6B in buffer A and enzyme was eluted with a 1 l gradient of 0–0.4 M NaCl in buffer A at a flow rate of 6 ml/min. Fractions of 12 ml were collected.

2.4. Amino acid sequencing

The MC column eluate was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and proteins (from ~0.5 to 5 μg) were excised from a gel that was lightly stained with Coomassie blue. The gel slices were washed in Milli-Q water (5×1 ml) for 1 h, brought to near dryness by rotary evaporation, suspended in 250 μl buffer B (50 mM Tris-HCl, pH 8.0, 0.01% alkylated Triton X-100) containing 1 μg of alkylated trypsin (Boehringer) and incubated for 20 h at 30°C. The supernatant was removed and a further 250 μl buffer B without trypsin was added and incubated overnight. The combined supernatants were applied to a 150×2.1 mm Vydac C18 column equilibrated in 0.1% trifluoroacetic acid (TFA) attached to a Applied Biosystems 140B high performance liquid chromatography (HPLC) system. The column was developed with a linear acetonitrile gradient in 0.09% TFA with an increase in acetonitrile concentration of 0.5% per min. A_{214} was recorded with an on-line monitor. The flow rate was 0.2 ml/min and fractions were collected manually. Selected peptides were sequenced on an Applied Biosystems 476A protein sequencer.

3. Results

Phosphorylase that has been phosphorylated on Ser-14 by phosphorylase kinase is a useful substrate for measuring members of the PPP1 and PPP2A (but not PPP5) subfamilies, whereas casein that has been phosphorylated by protein kinase A is a substrate for PPP2A and PPP5 (but not PPP1) [22–24]. The MC-sensitive phosphorylase phosphatase (PhP) activity in a cauliflower curd homogenate is distributed among soluble, membrane and salt-extractable fractions (not shown). In the present study, we prepared a soluble extract and concentrated the PhP activity by PEG precipitation for MC affinity chromatography. Only ~30% of the PhP activity in the PEG fraction bound to the MC column and increasing the time of incubation or column volume made no difference to the proportion of enzyme that bound. The recovery of PhP activity from MC-Sepharose was ~20% of which 97% was inhibited by the specific inhibitor of PP1, inhibitor 2. The column eluate showed three major bands on SDS-PAGE of ~35, 55 and 65 kDa and many minor bands. The 35 and 55 kDa bands bound [^{125}I]MC-YR, demonstrating that these proteins contained catalytic domains of the PPP family of PPs (not shown).

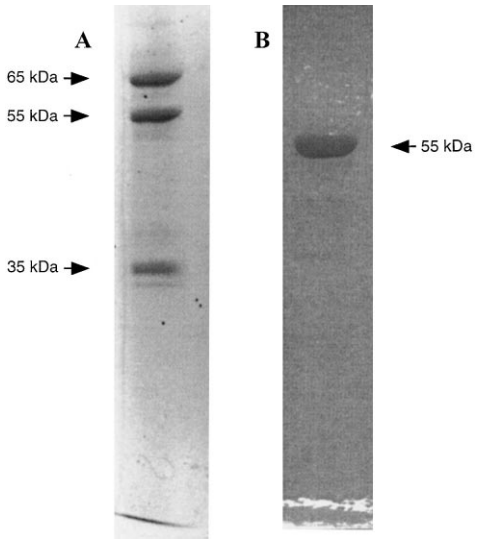

Fig. 1. SDS-PAGE of cauliflower proteins purified by MC-Sepharose chromatography. Coomassie blue-stained SDS-polyacrylamide (12%) gels of protein purified by MC-Sepharose chromatography of (A) a 0–17% PEG fraction of cauliflower extract and (B) the 60–150 mM NaCl elution pool from DEAE-Sepharose chromatography of a 0–17% PEG fraction of cauliflower extract.

Amino acid sequencing of peptides derived from in-gel tryptic digests identified the 35, 55 and 65 kDa proteins as being closely related to PP1, PP5 and an A-regulatory subunit of PP2A (Fig. 2). Peptides that aligned with sequences from both the catalytic and tetratricopeptide (TPR) repeat domains of human PP5 were identified (Fig. 2).

The mammalian PP5 has recently been found to have a very low casein phosphatase activity that is stimulated by arachidonic acid [23,24]. The MC-Sepharose eluate isolated from the cauliflower extract contained no detectable inhibitor 2-insensitive casein phosphatase activity, but activity could be detected in the presence of 200 μM arachidonic acid. In order to purify PP5 sufficient, free of PP1, for further characterisation of its regulatory properties, a cauliflower extract was fractionated by DEAE-anion-exchange chromatography and assayed for casein phosphatase activity in the presence and absence of arachidonic acid. A single peak of arachidonic acid-dependent casein phosphatase activity was detected at 80–100 mM NaCl, which was separated from the major peaks of PP1- and PP2A-like PhP activity, at 180–250 mM and 250–320 mM NaCl, respectively. MC-Sepharose chromatography of the DEAE-column fractions containing the arachidonic acid-dependent casein phosphatase activity yielded ~20 μg

A. 35 kDa protein – catalytic subunit of PP1

```
      235   244
TOPP1 GVSYTF GADK
TOPP2 GVSYTF GPDK
TOPP3 GVSYTF GADK
TOPP4 GVSYTF GPDK
TOPP5 GVSYTF GADK
TOPP6 GVSYTF GSDI
TOPP7 GVSYTF GADK
TOPP8 GISCTF GADK
      GVSYTF GPDK
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B. 55 kDa protein – PP5

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      1                                                  50
  ARA ..... ..... ..... ..... .....
HUMAN MAMAEGERTE CAEPPRDEPP ADGALKRAEE LKTQANDYFK AKDYENAIKF
S.POM ..... ..... ...MAKEALE LKNEANKFLK EGHIVQAIDL
      51                                                 100
  ARA ..... ..... ..... ..... .....
HUMAN YSQAIELNPS NAIYYGNRSL AYLRTECYGY ALGDATRAIE LDKKYIKGYY
S.POM YTKAIELDST NAILYSNRSL AHLKSEDYGL AINDASKAIE CDPEYAKAYF
        AIELNGN NAVYWANR       XEEYGS AIQDASK
      101                                                150
  ARA ..... ..... ..... ..... .....
HUMAN RRAASNMALG KFRAALRDYE TVVKVKPHDK DAKMKYQECN KIVKQKAFER
S.POM RRATAHIAIF QPKEAVGDFR  KALALAPSDP AARKKLRECE QLVKRIRFQE
      151                                                200
  ARA ..... ..... ..... ..... .....
HUMAN AIAGDEHKRS VVDSLDIESM TIEDEYSGPK LEDGKVTISF MKELMQWYKD
S.POM AIHNTEP.PS PLANINIEDM DIPSDYDGVI LEK.QITKEF VEDMKERFCQ
                  IEGEEV TLDFVK
                  IEGEEV TLEFVK
      201                                                250
  ARA ..... ..... ..... ..... .....
HUMAN QKKLHRKCAY QILVQVKEVL SKLSTLVETT LKETEKITVC GDTHGQFYDL
S.POM GKKLPLKFAY SILRDLKELL EKTPSLIDIP VKGDETLVIC GDTHGQYFDL
          SAY QIVLRVK
      251                                                300
  ARA ..... ..... ..... ..... .....
HUMAN LNIFELNGLP SETNPYIFNG DFVDRGSFSV EVILTLFGFK LLYPDHFHLL
S.POM LNIFKLHGPP SPTNKYLFNG DFVDRGSWST EVAFTLYAYK LLYPDAVFIN
                            XISV AEIDFHYIIK
      301                                                350
  ARA ...RESKSMN KIYGFEGEVR SKLSEKFVDL FAEVFCYLPL AHVINGKVFV
HUMAN RGNHETDNMN QIYGFEGEVK AKYTAQMYEL FSEVFEWLPL AQCINGKVLI
S.POM RGNHETDDMN KVYGFEGECR SKYNERTFNI FSETFSLLPL GSLISDSYLV
              IYGFEGEVR R                                IFV
      351                                                400
  ARA VHGGLFSVDG VKLSDIRAID RFC..EPPEE GLMCELLWSD PQPLPGRGPS
HUMAN MHGGLFSEDG VTLDDIRKIE RNR..QPPDS GPMCDLLWSD PQPQNGRSIS
S.POM VHGGLFSDDN VTLDQLRNID RFSKKQPGQS GLMMEMLWTD PQPAPGRGPS
      VHGGLFSVDG VKWWL
      401                                                450
  ARA KRGVGLSFGG DVTKRFLQDN NLDLLVRSHE VKDEGYEVEH DGKLITVFFA
HUMAN KRGVSCQFGP DVTKAFLEEN NLDYIIRSHE VKAEGYEVAH GGRCVTVFSA
S.POM KRGVGLQFGP DVSKRFCEAN GLKAVIRSHE VRDQGYEVEH DGYCITVFSA
                            AHE VKDEGYE
      451                                                501
  ARA PNYCDQMGNK GAFIRFEAPD MKPNIVTFSA VPHPD..... .....
HUMAN PNYCDQMGNK ASYIHLQGSD LRPQFHQFTA VPHPNVKPMA YANTLLQLGMM
S.POM PNYCDSTGNL GAVIKVKE.D MELDFHQFEA VPHPNIRPMA YANGLLSAI..
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C. 65 kDa protein – A-regulatory subunit of PP2A

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      190      200   279    287   515     524   537              551
PP2AA L LQLVESTFLI   TD LVPAYVR   FLPVVV EASK   LLQS LIPIVDQSVVD
 RCN1 F ATTVESTFLI   TD LVPAYVR   FLPVVV EASK   LLQS LIPIVDQSVVD
 pDF1 F AATVESAHLK   TE LVPAYVR   LLPVVM TASK   VLQS LIPIVDQSVVE
 pDF2 F AATIESAHLK   TD LVPAYAR   LLPAVI TASK   MMQS LIPIVDQAVVE
      F AATIESAHLK   TE LVPAYVR   LLPVVM TGAK   VLQS LIPIVDQSVVE
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Fig. 2. Sequences of peptides derived from amino acid sequencing of purified MC-binding proteins. The 35, 55 and 65 kDa bands (Fig. 1) were excised from a lightly stained SDS-polyacrylamide gel. Bands were digested with trypsin and the peptides separated by reverse-phase HPLC. Peptide sequences from each of the indicated bands are underlined in single letter code. An 'X' means that no residue could be identified at this position. Where two sequences were identified in roughly equimolar amounts in the same position, both are shown one above the other. Sequences from peptides isolated from the bands are shown in alignment with the closest matches from sequences in the data bases, identified by their names and accession numbers. (A) Of the eight *Arabidopsis* PP1 isoforms (TOPPs) [39], the peptide obtained was a perfect match with predicted protein sequences from *Arabidopsis* TOPP2 and TOPP4. Numbering is that of TOPP1. (B) The peptides are aligned with deduced amino acid sequences of full-length human PP5 (P53041), *Schizosaccharomyces pombe* PP5 (AL022019) and an *Arabidopsis* EST (AF032879) and residues in bold conform to the TPR consensus sequence [1]. Numbering is for human PP5. Obvious matches could not be found for several other peptide sequences obtained from the 55 kDa band, namely, ISVAEIDFHYIIK, RVAESIDYHTIEVEPQ, FLEDNNLDLVVRWPQW, SAFIR, FSNAIDLYTIC, MVGEEAGGAPVXE, FIQV, FILQILPYLQAISVQ, PQAAGLTVDG, EPGD. (C) Of the published *Arabidopsis* sequences of regulatory A-subunits of PP2A, the peptides obtained were more similar to the *Arabidopsis* pDF1 (S51808) than to pDF2 (S51809) [16], PP2AA (S69215) or RCN1 (U21557) [17]. Numbering is for PP2AA.
←

pure PP5, representing a yield of 80% and a purification factor of 500-fold from the MC column load.

The activity of the plant PP5 was stimulated ~10-fold by arachidonic acid to ~300 mU/mg, with a half-maximal stimulation occurring at ~100 μM arachidonic acid. At 200 μM, oleic acid and linoleic acid also activated the enzyme 4–10-fold. In contrast, arachidic acid arachidonyl methyl ester, oleic acid methyl ester, myristic acid, caproic acid and the plant lipid jasmonic acid (JA) (see Section 4) had little or no effect.

4. Discussion

Here, we have purified a plant PP1, an A-regulatory subunit of PP2A, and PP5, demonstrating the value of MC-Sepharose for purifying PPs other than PP1 and providing the first report of PP5 in plants. The MC-Sepharose eluate also contained a further 20–30 minor proteins and preliminary experiments suggest that some of these may be PP1-binding proteins (not shown).

The MC-Sepharose affinity column has a high capacity and affinity for binding PP2A and the low recovery (20%) of PhP activity is largely due to our failure to dissociate significant active PP2A catalytic (C) subunit, which is similar to our previous experience with mammalian PP2A [19,20]. However, we have shown here that MC-Sepharose can be used to purify the regulatory A-subunit of PP2A. The purified plant A-sub-

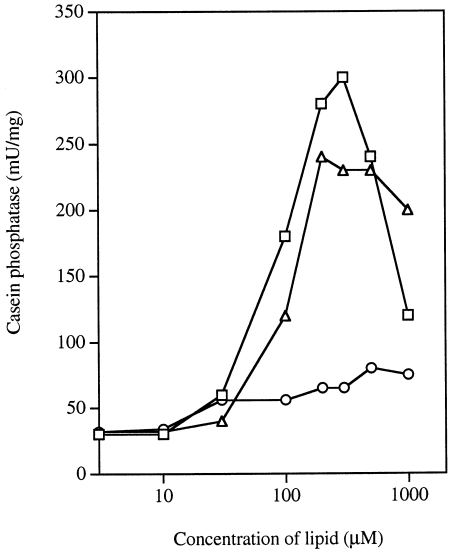

Fig. 3. Effects of lipids on casein phosphatase activity of cauliflower PP5. PP assays contained 8 ng of PP5 (~5 nM). Lipids are arachidonic acid (squares), linoleic acid (triangles) and JA (circles). Data are the means of duplicates that varied by less than 5% and similar results were seen in three experiments, except that an apparent activation was seen with one preparation of JA (see Section 4).

unit could bind to mammalian PP2AC and the resulting complex was stable to gel filtration (not shown), supporting the suggestion that the core AC complex of PP2A is conserved.

The finding that the percentage of PhP in the crude cauliflower extract that bound to the MC-Sepharose was always ~30%, despite increasing the binding time and column capacity, indicated that, similar to mammalian extracts, there may be endogenous PP inhibitors in the plant extract that compete with MC for binding to the PPs. Consistent with this suggestion, partially purified PP5 bound to and was recovered from the MC-Sepharose with a yield of >80%. Actually, in view of the fact that the TPR domain is thought to shield the active site [23] that overlaps with the MC-binding site of the PPP enzymes [25], it was somewhat surprising to find that PP5 could be purified by MC-Sepharose at all.

The literature on mammalian PP5 does not give obvious clues about functions for PP5 in plants because mammalian PP5 apparently interacts via its TPR domain with many proteins, of which only a few have known homologues in plants. Thus, mammalian PP5 has been reported to interact via the TPR domains with the atrial natriuretic peptide receptor [26], a complex of the glucocorticoid receptor and hsp90 [27,28], two TPR-containing proteins (CDC16, CDC27) of the anaphase-promoting complex [29] and the human protein hCRY2, a member of the photolyase/UV-B photoreceptor family which is also represented in plants and bacteria [30]. In mammalian cells, PP5 has been reported to be located mainly in the nucleus where it becomes concentrated in the nucleolus in cells treated with α-amanitin at concentrations that inhibit RNA polymerase III, suggesting a role for PP5 in regulating ribosomal RNA transcription [31]. Experiments in which cellular expression of PP5 was inhibited using antisense oligonucleotides have implicated PP5 as acting upstream of p53 to regulate the induction of the p21 (WAF1/Cip1) inhibitor of cyclin-dependent kinases and mediate growth arrest [32].

Other plant proteins containing TPR repeats have been identified (for example, [33]) and found as expressed sequence tags (ESTs) in the *Arabidopsis* genome sequencing projects. However, the PP5 activity in a crude cauliflower extract chromatographed on gel filtration at ~55 kDa (not shown), so we have no evidence that it forms a high affinity complex with other plant proteins.

The plant PP5 was activated by arachidonic acid and other polyunsaturated fatty acids with a similar specificity for long chain, unsaturated, charged lipids, similar kinetics and specific activity of the activated enzyme to the human and bovine forms of the enzyme [23,24]. Arachidonic acid produced by fungi is recognised by plants as an elicitor of defence responses [34–36]. However, arachidonic acid is not thought to be an endogenous regulatory molecule in plants. The plant octadecanoid pathway, which is analogous to arachidonic acid synthesis in mammalian cells, produces JA (12:1) from linolenic acid. JA and its derivatives mediate changes in gene expression in response to environmental and developmental processes [37]. Certain JA-mediated changes in gene expression in response to wounding are blocked by okadaic acid, demonstrating a requirement for PP activity [38]. In preliminary experiments, the purified plant PP5 appeared to be activated by JA (not shown), but this effect was not reproduced with three other batches of the lipid (Fig. 3), indicating that the initial observation may have been due to a contaminant.

The ability to purify PPs and their regulatory subunits by MC-Sepharose chromatography will be a valuable contribution to characterising the structure and physiological properties of these central regulatory enzymes in plants.

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