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Expression of the pea gene $PsMT_A$ in *E. coli*

Metal-binding properties of the expressed protein

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The pea (*Pisum sativum* L.) gene $PsMT_A$ has an ORF encoding a predicted protein with sequence similarity to class I metallothioneins (MTs). To examine the metal-binding properties of the $PsMT_A$ protein it has been expressed in *E. coli* as a carboxyterminal extension of glutathione-S-transferase (GST). Metal ions were associated with the expressed protein when purified from lysates of *E. coli* grown in metal supplemented media. The pH of half-dissociation of Zn, Cd and Cu ions from the recombinant fusion protein was determined to be 5.35, 3.95 and 1.45 respectively, compared with equivalent estimates of 4.50, 3.00 and 1.80 for equine renal MT.

$PsMT_A$; $PsMT_A$ protein; *Pisum sativum* L.; Metallothionein; Metal metabolism

1. INTRODUCTION

The E_c protein isolated from wheat germ is known to bind Zn [1] and is defined as a higher plant class II metallothionein (MT) [2]. However, MT (class I or II) has not yet been shown to occur in vegetative plant tissue. The pea (*Pisum sativum* L.) gene, $PsMT_A$, has sequence similarity to class I MT genes [3]. Related cDNAs have also been isolated from libraries prepared from poly(A)$^+$ RNA purified from roots of *Mimulus guttatus* [4] and several unpublished sequences are also now known for homologous genes isolated from other higher plants. $PsMT_A$ transcripts have been detected in pea roots 14 days after germination [3] but $PsMT_A$ protein and/or products of related higher plant genes have not yet been isolated and sequenced. Confirmation that $PsMT_A$ is a plant MT gene requires the characterisation of its putative product. MT genes from a number of different organisms have been expressed in *E. coli*, and also in yeast, and the proteins shown to form the correct conformation to associate with metal ions [5–10]. In some cases MT has been demonstrated to bind metal ions when expressed as a fusion with another protein [6,7,10]. We describe here the expression in *E. coli* of $PsMT_A$ as a carboxy-terminal extension of glutathione-*S*-transferase, and report the metal-binding characteristics of the expressed protein.

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2. MATERIALS AND METHODS

2.1. *In vitro amplification, cloning and sequence analysis of* $PsMT_A$ *coding region*

Two oligonucleotide primers 5'GGCGAATTCGTCTGGATGTGGTTGTGG 3' and 5'GGCGAATTCATTTGCAGTTGCAAGGGTC 3' were synthesized using an Applied Biosystems 381A DNA synthesizer. The 3' regions of the primers correspond to opposite ends of the $PsMT_A$ protein coding region (excluding the 5' ATG) such that they will associate with double stranded template DNA to give proximal 3' ends. The 5' regions contain *Eco*RI restriction endonuclease recognition sites to facilitate cloning (in frame) into the glutathione-*S*-transferase (GST) fusion-protein expression vector, pGEX3X (Pharmacia).

Poly(A)$^+$ RNA was isolated from the roots of pea plants (*Pisum sativum* L., cv Feltham First) and used for the synthesis of cDNA [11]. Polymerase chain reaction (PCR) was performed essentially as described previously [12] using 15 ng of total cDNA as template. Reactions were subjected to 33 cycles of the following series of temperatures and times: 92°C for 1.5 min (denaturation), 50°C for 2 min (annealing), 72°C for 2 min (extension) using a Hybaid Intelligent Heating Block. Ten microlitres of reaction mixtures were analysed by electrophoresis on 2% agarose gels. Diagnostic-sized amplification products were electro-eluted from agarose gel slices and the DNA recovered from solution by binding to silica fines. Fragments were digested with *Eco*RI and then ligated into the *Eco*RI site of pGEX3X.

The sequence and orientation of the cloned $PsMT_A$ fragment in pGEX3X (pGPMT3) was verified by sequencing in both directions using the PCR oligonucleotide primers. DNA sequencing was performed by modified dideoxy termination (Dye Deoxy™ Termination: Applied Biosystems) and analysed using an Applied Biosystems 370A DNA sequencer.

2.2. *Expression, purification and characterisation of the recombinant* $PsMT_A$ *protein*

An overnight culture of *E. coli* JM101 containing pGPMT3 was diluted 1:10 (v/v) in fresh LB medium supplemented with 50 μg/ml ampicillin. Cells were grown at 37°C for 45 min and metal (Zn, Cd or Cu) added to a final concentration of 500 μM. Cells were grown for a further 15 min at 37°C before expression of the fusion protein was induced by addition of isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 1 mM. Cells were grown for an additional 4

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h at 37°C, pelleted by centrifugation at 5000 × *g* for 15 min and resuspended in 1% of the original volume of an ice-cold solution of 1% (v/v) Triton X-100, 150 mM NaCl, 16 mM NaH_2PO_4, 4 mM Na_2HPO_4, pH 7.3. Cells were lysed by mild sonication at 4°C and the fusion protein purified from the supernatant by single-step affinity chromatography using glutathione-Sepharose-4B (Pharmacia) [13]. The bound fusion protein was eluted with 3 bed volumes of 5 mM glutathione in 50 mM Tris-HCl, pH 8.0. Column eluant was passed through columns of Sephadex G-25 (PD-10 Pharmacia) and protein content estimated using a Coomassie blue-based reagent (Bio-Rad) and bovine serum albumin (BSA) as a standard. Metal concentration of samples was determined by atomic absorption spectrophotometry. Proteins were resolved on 15% SDS-PAGE gels [14] and visualised following Coomassie brilliant blue staining.

The polylinker of pGEX3X contains a specific protease recognition sequence for blood coagulation factor Xa [15] which facilitates cleavage of the cloned protein from GST. $PsMT_A$ protein (purified from cells grown in Zn-supplemented media) was cleaved from GST while the fusion protein was associated with glutathione-Sepharose-4B [16]. Column eluant containing factor Xa and $PsMT_A$ protein was subsequently fractionated on Sephadex G-50. Fractions (2.5 ml) were collected and analysed for protein and metal. An aliquot (1 ml) of the Zn-peak fraction was vacuum blotted onto Immobilon-P (Applied Biosystems) and subjected to amino acid sequence analysis (ABI model 477A gas-phase microsequencer) using standard operating procedures.

2.3. *Analysis of metal-binding properties of the expressed protein*

Aliquots (350 μl) of $PsMT_A$–GST fusion protein (purified from cells supplemented with either Cd, Zn or Cu and containing between 15 and 50 nM of the respective metal) in 0.02 M potassium phosphate (pH 8.91) were incubated for 1 h at room temperature with 2.15 ml of the following buffers: 0.05 M KCl/HCl (pH 1.10–1.37); 0.05 M glycine/HCl (pH 2.04–2.91); 0.04 M sodium acetate/acetic acid (pH 3.6–5.43); 0.02 M KH_2HPO_4/orthophosphoric acid (pH 6.03–pH 9.44); and the pH of the mixed solutions measured. After 1 h samples were fractionated on columns of Sephadex G-25 (PD-10 Pharmacia) equilibrated with the same buffers. Metal ions eluting in the void volume and total volume of each column were quantified by atomic absorption spectrophotometry and the proportion which eluted in the void volume was calculated. The data is expressed as a percentage of the values obtained at the highest pH.

Preparations of Zn–$PsMT_A$–GST fusion protein were incubated with a two-fold molar excess (with respect to Zn) of either Cu or Cd, unbound metal removed by gel filtration (PD-10) and the above procedure repeated to estimate the pH of half-dissociation of in vitro associated metal ions. The pH of half-dissociation of Cd and Zn was also estimated for a commercial preparation (Sigma Chemical Co.) of equine renal (Cd, Zn)-thionein. In addition, equine (Cd, Zn)-thionein was incubated with a two-fold molar excess (with respect to total bound Zn and Cd) of Cu and the pH of half-dissociation determined.

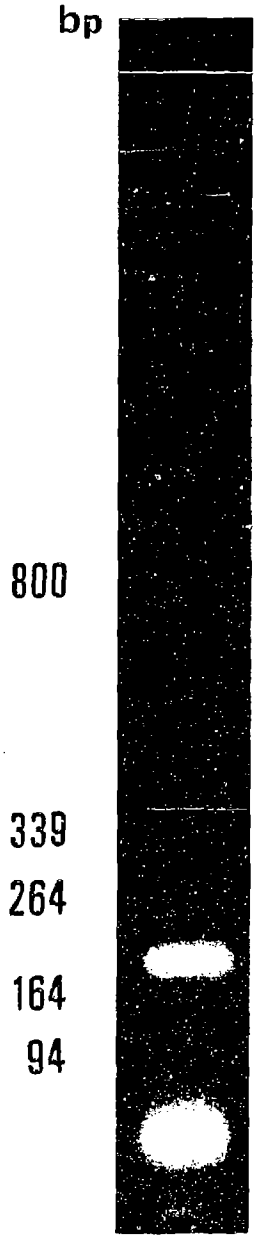

Fig. 1. Visualisation of PCR products on an agarose gel. Amplification conditions were as described in section 2.

3. RESULTS AND DISCUSSION

Amplification products of the predicted size (235 bp), corresponding to the protein coding region of $PsMT_A$ plus additional primer sequences, were detected following agarose gel electrophoresis of PCR products (Fig. 1). These products were inserted into the expression vector pGEX3X, as described in section 2, to produce plasmid pGPMT3.

A protein corresponding to the size of GST (26.5 kDa) was detected in crude lysates of induced JM101 cells containing the vector pGEX3X (Fig. 2, lane 2). A unique protein, corresponding to the predicted size (ca. 34.5 kDa) of the $PsMT_A$–GST fusion, was detected in crude lysates of cells transformed with plasmid pGPMT3 (Fig. 2, lanes 5 and 6). A slightly larger unique protein was detected in lysates of cells transformed with plasmid pGPMT4 (Fig. 2, lanes 3 and 4) which contains the $PsMT_A$ insert in the reverse orientation. This would be anticipated due to the absence of in-frame stop codons within the reversed $PsMT_A$ sequence. After purification of the $PsMT_A$–GST fusion protein by affinity chromatography using glutathione-Sepharose 4B, a single protein of ca. 34.5 kDa was detected in fractions eluted with buffer containing 5 mM glutathione (Fig. 2, lane 8). This protein corresponded in size to the most abundant protein observed in crude lysates of cells containing this construct.

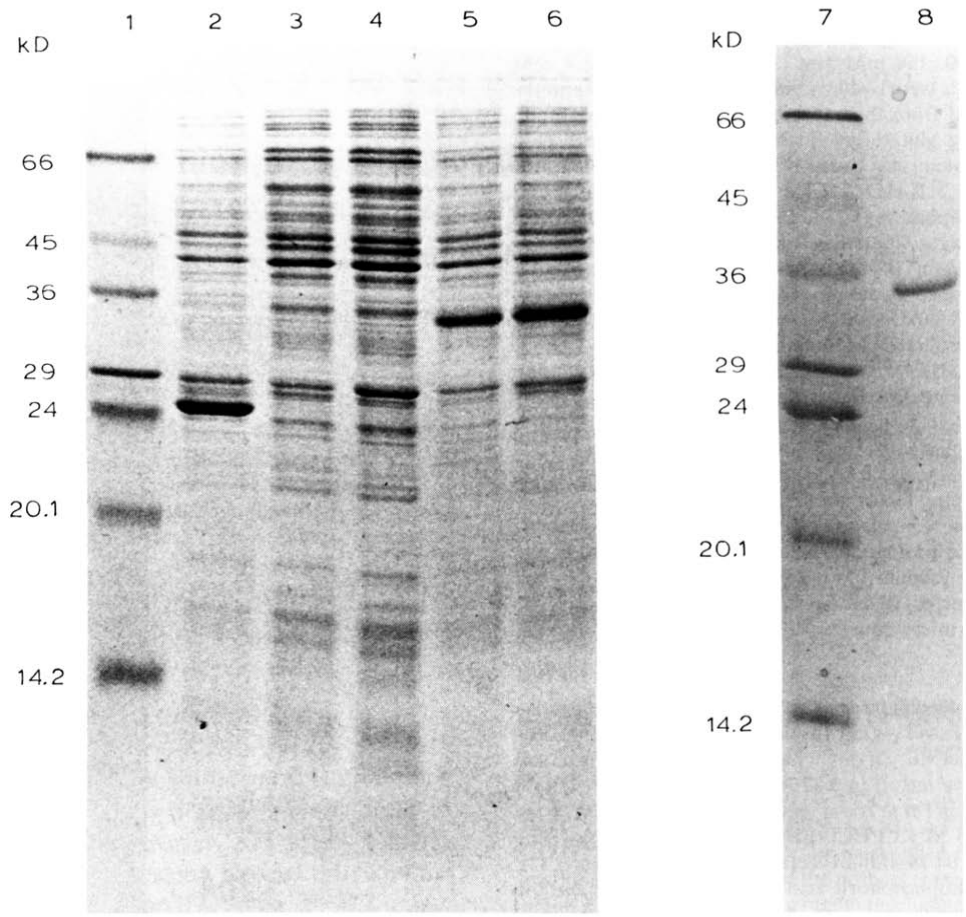

Fig. 2. Analysis of *E. coli* cell extracts by SDS-PAGE on 15% gels. Gels were stained with Coomassie blue. Origin of protein samples: molecular weight markers (lanes 1 and 7); crude lysate from IPTG-induced cells containing pGEX3X (lane 2); crude lysate from IPTG-induced cells containing pGPMT4 grown in the presence (lane 4) or absence (lane 3) of 500 μM Zn; crude lysate from IPTG-induced cells containing pGPMT3 grown in the presence (lane 6) or absence (lane 5) of 500 μM Zn; glutathione-Sepharose-purified extract from IPTG-induced cells containing pGPMT3 grown in the presence of 500 μM Zn (lane 8).

The bound metal content of GST and the $PsMT_A$–GST fusion was determined for protein purified from *E. coli* grown in media supplemented with Cd, Cu or Zn (Table I). Greater association of metal ions with the fusion protein, than with GST alone, suggests that metal ions bind to the $PsMT_A$ portion of the protein.

Table I

Metal content of GST and GST–$PsMT_A$, purified from lysates of cells grown in media supplemented with 500 μM Zn, Cd or Cu

Protein	Zn:Protein	Cd:Protein	Cu:Protein
GST	0.16:1	0.69:1	0.65:1
	0.56:1	0.47:1	1.20:1
	0.43:1	0.56:1	0.94:1
$PsMT_A$–GST	4.27:1	4.10:1	3.53:1
	7.80:1	4.41:1	3.21:1
	5.98:1	4.82:1	2.94:1

Data are shown for extracts from three replicate cultures.

However, differences in the reactivities of GST, $PsMT_A$ and BSA in the protein assay will cause inaccuracies in the estimated stoichiometries. Furthermore, multiple bands were detected following analysis by SDS-PAGE of $PsMT_A$–GST purified from lysates of cells grown in media supplemented with 500 μM Cu. The presence of contaminants will reduce the apparent ratio of Cu fusion protein.

To confirm that Zn associates with the $PsMT_A$ portion of the fusion protein, the protein was cleaved with factor Xa while bound to glutathione-Sepharose. This releases $PsMT_A$ plus a short extension (^{N}Gly-Ile-Pro-Gly-Asn-Ser^C) prior to the N-terminal Ser residue of $PsMT_A$, leaving the GST portion of the protein retained on the matrix. Column eluate was fractionated on Sephadex G-50. Fig. 3 shows that Zn is associated with a low mol. wt. protein released by factor Xa. The higher mol. wt. protein excluded from the Sephadex G-50 matrix is assumed to be factor Xa. Amino acid sequence analysis of an aliquot of fraction 31 (Zn-peak fraction with estimated Zn:protein of 11.5:1, assuming a mol. wt.

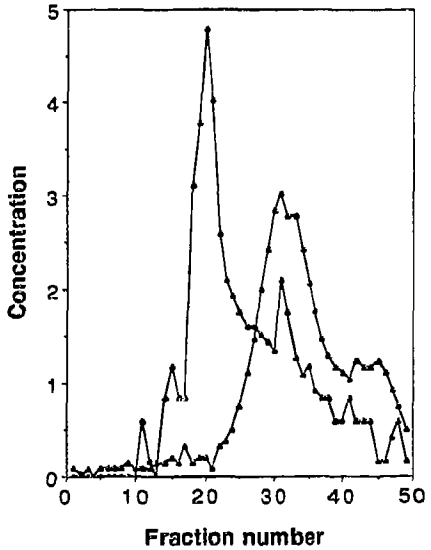

Fig. 3. Gel filtration chromatography on Sephadex G-50 of GST-$PsMT_A$ fusion protein eluted from glutathione-Sepharose-4B following incubation with factor Xa. Protein (in μg/ml) was determined by dye-binding assay (△) and Zn (in μM) was quantified by atomic absorption spectrophotometry (▲).

of 8000) gave the sequence of the N-terminal extension of $PsMT_A$ but the amino acid yield for the predicted continuation of this sequence is low. This is likely to be due to the presence of two adjacent Ser residues, which typically reduce sequencing yields (Table II). In addition, a second sequence (^{N}Gly-Val-Gly-Pro-Ala-Lys-

Table II

Amino acid sequence analysis of fraction 31 (Zn peak) after gel filtration on Sephadex G-50 of cleaved $PsMT_A$

Cycle	1	2	3	4	5	6	7	8	9	10	11	12
Ala	2.3	−3.6	−2.6	4.3	*31.5*	0.0	6.4	2.2	1.0	−3.2	−0.2	*20.2*
Arg	4.6	0.9	−0.2	−0.6	1.1	2.2	0.0	0.2	0.3	−0.4	−0.2	0.4
Asn	6.1	0.0	−0.7	0.0	**39.9**	4.0	1.4	0.7	0.0	−1.0	0.9	0.3
Asp	9.5	0.0	−0.3	−0.1	4.4	0.9	0.1	−0.1	−0.1	−0.7	0.3	2.5
Cys	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	**−1.0**	−1.0	**1.0**	−1.0
Glu	−0.5	4.7	0.0	9.0	9.3	0.0	−1.8	3.1	0.0	*5.5*	0.9	−0.8
Gln	−0.3	8.6	15.3	1.4	1.0	1.6	0.5	*22.4*	3.6	0.0	0.2	0.1
Gly	***105.5***	0.0	*41.4*	**43.0**	1.6	3.0	−0.7	**8.0**	−0.2	**1.4**	*13.5*	**11.0**
His	−0.4	5.1	0.0	−0.3	0.1	0.6	1.1	0.1	0.1	0.3	−0.3	−0.3
Ile	−0.2	**86.8**	0.0	0.0	−0.3	0.9	*27.3*	5.5	1.2	0.0	−0.6	1.1
Leu	−0.3	8.7	0.0	−0.2	1.0	−0.0	0.2	0.1	0.1	−0.5	−0.4	−0.2
Lys	11.1	0.4	0.0	−0.4	−0.3	*18.8*	1.6	0.0	0.5	−0.2	−0.5	0.2
Met	7.2	0.0	−0.5	−0.4	5.2	4.7	0.0	−0.2	3.4	0.0	−0.4	−0.4
Phe	9.9	0.0	−0.3	8.6	0.0	−0.3	−0.3	−0.4	*19.7*	2.2	1.0	0.6
Pro	−1.2	1.6	**86.3**	*34.2*	6.7	5.2	4.0	3.2	6.0	1.1	0.0	0.5
Ser	8.0	0.0	0.1	−0.1	−0.7	**8.8**	**7.5**	0.6	0.4	0.1	−0.8	−0.4
Thr	8.5	1.3	−0.1	−0.4	−1.5	−4.7	4.1	−0.4	1.6	2.2	8.0	0.5
Trp	3.6	0.0	0.0	−0.3	−0.3	−0.4	−0.0	−0.1	0.0	0.0	0.5	0.5
Tyr	0.0	0.1	0.1	−0.0	−0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0
Val	17.7	*39.3*	0.0	0.0	−0.2	0.1	0.4	−0.1	−0.3	−0.5	1.1	0.8

The yield (pM) of each amino acid is shown at each cycle. The sequence of an internal region of $PsMT_A$ (italics) and the N-terminus of the cleaved protein (bold) are underlined.

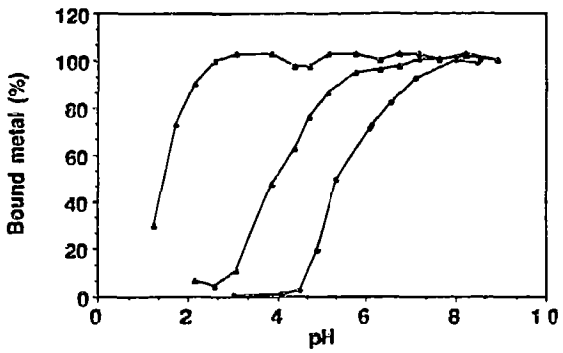

Fig. 4. Hydrogen ion competition of metal-binding to $PsMT_A$-GST fusion protein. The data is expressed as a proportion of metal bound at pH 8.9. Protein was purified from *E. coli* grown in media supplemented (500 μM) with either Zn, Cd or Cu. Aliquots of purified (via glutathione-Sepharose 4B) protein were incubated for 1 h at the indicated pH. Free and bound Zn (◇), Cd (△) and Cu (▲) were resolved by gel filtration on columns of Sephadex G-25 equilibrated with the appropriate buffer.

Ile-Gln-Phe-Glu-Gly-Ala^{C}) was detected which corresponds to an internal region of $PsMT_A$ protein and is presumed to have arisen due to proteolytic cleavage. The presence of cleaved $PsMT_A$ polypeptides in fraction 31 may alter the estimated Zn:protein stoichiometry.

Estimations of the pH at which 50% of metal is dissociated is a criterion used to distinguish MT from non-MT metal-binding proteins [17]. The pH of half-dissociation of Zn, Cd and Cu from $PsMT_A$-GST, purified from *E. coli* grown in media supplemented with the respective metal ions, was estimated to be 5.25, 3.95 and 1.45, respectively (Fig. 4). SDS-PAGE indicated that fusion protein isolated from cells supplemented with Cu was impure. To overcome this problem Cu was bound in vitro to fusion protein purified from cells supplemented with Zn which was resolved as a single band on SDS-PAGE (Fig. 2). Following removal of unbound Cu by gel filtration (PD-10), the stoichiometry (Cu:protein) was estimated to be 6.17:1. The pH of half-dissociation of in vitro bound Cu was estimated to be 1.50 which is similar to the value (1.45) obtained previously for Cu bound in vivo. The pH of half-dissociation of in vitro bound Cd (stoichiometry estimated to be 5.76:1) was 3.95 which is also in agreement with estimates obtained using metal associated with protein in vivo. The pH of half-dissociation of Zn, Cd and Cu from equine MT was determined to be 4.50, 3.00 and 1.80, respectively. Previously reported values for the pH of half-dissociation of Zn and Cd from equine MT agree with those estimated here [18,19].

Plants synthesize the metal-binding polypeptide poly(γ-glutamylcysteinyl)glycine, $(\gamma EC)_nG$ (which has been referred to as phytochelatin, cadystin and class III MT), in response to elevated concentrations of certain toxic trace metals [20–27]. The pH of half-dissociation of Cd from complexes of $(\gamma EC)_nG$ isolated from *Schizosaccharomyces pombe* was 4.0 and 5.4, depending upon whether or not the complexes contained additional inor-

ganic sulphide, while the pH of half-dissociation of Cu was ca. 1.3 [28]. Values for the displacement of Cd from these polypeptides isolated from tobacco ranged from 5 to 5.8 [29]. In comparison to analyses of equine MT and $(\gamma EC)_nG$ the data presented here suggest that $PsMT_A$ also has relatively high affinities for certain metal ions, supporting the contention that it is a plant MT. Further experiments are required to detect the translational product of *$PsMT_A$* in planta and to examine whether the metal-binding properties of the GST- $PsMT_A$ fusion protein are similar to those of the native $PsMT_A$ protein.

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