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QUARTERLY

Expression of *Lupinus luteus* cDNA coding for PR10 protein in *Escherichia coli*: Purification of the recombinant protein for structural and functional studies*

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The cDNA clones coding for two pathogenesis-related protein homologues of PR10 class, *Ll*PR10.1A and *Ll*PR10.1B, were identified in yellow lupin expression library of uninfected roots. The contribution of PR10 proteins to the overall mechanism of plant defence still remains unknown. In order to elucidate the structure and function of lupin PR10.1A protein, a substantial quantity of the protein was produced in an *E. coli* expression system using plasmids of pET-series: pET-3a and pET-15b, carrying the T7 promoter. Both plasmids with subcloned *Llpr10.1a* gene were overexpressed in *E. coli*, strain BL21(DE3)pLysS. The recombinant *Ll*PR10.1A protein, overproduced in bacterial cells transformed with the pET-3a/*Llpr10.1a* plasmid, was purified to homogeneity from the insoluble "inclusion bodies" by ammonium sulphate fractionation and two sequential chromatographic steps: ion-exchange chromatography on DE 52 cellulose followed by size exclusion chromatography on Superdex 75 FPLC column. The $(His)_6$ *Ll*PR10.1A protein overproducted in *E*. coli cells harbouring the pET-15b/*Llpr10.1a* plasmid was purified by chromatography on Ni^{2+}-charged His.Bind Resin. Western blot analysis with rabbit serum containing anti-*Ll*PR10.1A^{N} antibody revealed identical immunochemical properties of the two recombinant polypeptides and native *Ll*PR10.1A protein. The recombinant protein produced in pET-3a plasmid was renatured from its insoluble form, concentrated up to 22 mg/ml and submitted to crystallisation. However, the *Ll*PR10.1A protein expressed in pET-15b plasmid precipitated from the solution when at a higher concentration (10 mg/ml). This preparation was used at a lower concentration as an antigen for the preparation of polyclonal antibodies for immunochemical studies.

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Abbreviations: *Ll*PR10.1A^{N}, native *Lupinus luteus* pathogenesis-related protein of PR10 class; *Ll*PR10.1A^{E}, recombinant protein expressed in *E. coli* cells in pET-3a plasmid; $(His)_6$-*Ll*PR10.1A, recombinant protein expressed in *E. coli* cells in pET-15b plasmid as a fusion protein; *Llpr10.1a,* gene encoding *Ll*PR10.1A; pET-3a/*Llpr10.1a*, pET-3a expression plasmid carrying *Ll*PR10.1A encoding gene; pET-15b/*Llpr10.1*a, pET-15b expression plasmid carrying *Ll*PR10.1A encoding gene; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactopyranoside.

Two homologous proteins of PR10 class — *Ll*PR10.1A and *Ll*PR10.1B (Sikorski *et al.*, 1989; Sikorski, in preparation) and corresponding full-length cDNA clones (Sikorski *et al.*, 1996; Legocki *et al.*, 1997) were identified in yellow lupin. The predicted amino-acid sequences of the two protein homologues revealed a high degree of homology (76% identity, 90% similarity) indicating that genes encoding these proteins belong to a multigene family. It was shown that genes coding for *Ll*PR10 proteins are constitutively expressed in roots but not in the infected plant and their expression is down-regulated during the nodule development after the inoculation with symbiotic bacteria — *Bradyrhizobium*, sp. (*Lupinus*).

It was postulated that these acidic proteins of M_r 16–19000 are involved in the general plant defence mechanism, playing the role of a barrier against pathogen invasion, mechanical damage (wounding) or any environmental stress (chemical pollutants). However, the physiological function of pathogenesis-related proteins as well as their contribution to the pathogen defence still remain unknown. On the basis of a high amino-acid sequence homology and gene expression pattern similarity to ginseng ribonuclease, their ribonuclease activity in the defence reaction was suggested (Moiseyev *et al.*, 1994; 1995). Due to their structural similarity, PR10 proteins have been classified as ribonuclease-like PR proteins (Van Loon *et al.*, 1994).

Intracellular pathogenesis-related proteins have been shown to be ubiquitous in the plant kingdom (Walter *et al.*, 1990). They are structurally related to tree-pollen allergens and to major food allergens from celery and apple (Breiteneder *et al.*, 1989; 1995; Vanek-Krebitz *et al.*, 1995). The precise cellular localisation of PR10 proteins has not been determined but the absence of apparent signal peptides and the identity of cDNA-predicted amino-acid sequences to those obtained from protein sequencing classify them as cytosolic proteins (Walter *et al.*, 1990; Awade *et al.*, 1991). It has been reported by several laboratories that PR10 proteins accumulate around the sites of pathogen invasion or wounding (Schmelzer *et al.*, 1989; Somssich *et al.*, 1988; Warner *et al.*, 1992; 1993; Pinto & Ricardo, 1995; Breda *et al.*, 1996).

There are also suggestions that PR10 proteins play an important function in the plant development. They have been identified in seeds (Barratt & Clark, 1993; Warner *et al.*, 1994), developing roots, senescent leaves (Crowell *et al.*, 1992) and senescent nodules (Sikorski *et al.*, 1996; Legocki *et al.*, 1997), stems (Warner *et al.*, 1994) and different parts of flowers (Breiteneder *et al.*, 1989; Warner *et al.*, 1993; 1994; Constabel & Brisson, 1995; Swoboda *et al.*, 1994). It has recently been shown that birch pollen allergen *Bet v* 1 belonging to the PR10 protein class revealed RNase activity *in vitro* (Bufe *et al.*, 1996; Swoboda *et al.*, 1996).

In order to determine the three-dimensional structure and study the biological activity of PR10 protein I have used the T7 promoter/T7 RNA polymerase expression system to overproduce the *L. luteus* PR10.1A protein homologue using plasmids of pET-series (Studier *et al.*, 1990; Novagen Manual, 1993) and purified the renatured protein from "inclusion bodies" with a relatively high yield.

The identity of the purified recombinant protein with the native protein was confirmed by Western blot analysis using rabbit anti-*Ll*PR10.1A^N antibody.

MATERIALS AND METHODS

Bacterial strains and media. *E. coli* strains used were HMS174 (F^--*recAr*$^-_{K12}m^+_{K12}Rif^R$ and BL21 (DE3)pLysS (F^-*ompTr*$^-_B m^-_B$) (Studier *et al.*, 1990). The cDNA coding fragment of *Llpr10.1a* was introduced into *Nde*I and *Bam*HI cloning sites of pET-3a and pET-15b expression plasmids (Studier *et al.*, 1990; Novagen Manual, 1993). *E. coli* cells were grown at 37°C in LB medium supplemented with 100 µg ampicillin/ml and/or 50 µg chloramphenicol/ml. For overproduction of recombinant protein a large scale *E. coli* culture was grown in 5 l bioreactor, type Bioflo III (New Brunswick Scientific).

Chemicals, enzymes and chromatographic media. Salts of HPLC grade, glyc-

erol and Tris-base were purchased from Sigma; restriction endonucleases, T4 DNA ligase, TaqI polymerase, deoxynucleotides and DNA sequencing kit from Promega; antibiotics from Boehringer Mannheim; Bacto Tryptone and Yeast Extract were purchased from Difco.

The PCR primers were synthesized at the Laboratory of Dr. W. Markiewicz at the Institute of Bioorganic Chemistry, and purified by preparative 17% polyacrylamide gel electrophoresis at the presence of 7 M urea. The *Staphylococcus aureus* ^{125}I-labelled protein A was prepared at the Institute of Endocrinology, Medical University, Poznań and kindly provided by Dr. J. Sawicka. Rabbit anti-*Ll*PR10.1A^{N} antibody was produced at the Institute of Animal Endocrinology, Polish Academy of Sciences, Poznań, by Mrs G. Wieczorek. His.Bind Resin, His.Bind Buffer Kit and thrombin protease, restriction grade were Novagen products. The Superdex 75 HiLoad 16/60 FPLC column for size exclusion chromatography was from Pharmacia LKB; DE 52 cellulose, preswollen, was purchased from Whatman Company. Centricon-10 concentrators were from Amicon Company, U.S.A. Acrylamide was from Serva and bisacrylamide from Boehringer. Protein relative molecular mass standards for SDS/PAGE were purchased from Pharmacia LKB and Promega.

***Plant PR10.1A protein from L. luteus* (L.).** *L. luteus* PR10.1A protein was purified to homogeneity according to the procedure described by Sikorski *et al.* (1989) and used as an antigen to produce rabbit antibodies.

PCR amplification and mutagenesis of Llpr10.1a coding region of cDNA clone. The DNA region coding for *Ll*PR10.1A protein was copied and amplified from cDNA clone harbouring the Ll*pr10.1a* gene (Sikorski *et al.*, 1996). Mutagenesis of the internal *Nde*I and *Bam*HI recognising sequences was also performed in order to subclone the coding DNA fragment into unique restriction sites of the expression plasmids of the pET-series. The following primers were designed to create mutations and the *Nde*I and *Bam*HI sites at the 5′ and 3′ ends of the *Llpr10.1a* gene:

N: 5′ CGTCGC**CATATG**G-
GTATTTTTGCTTTCGAAAATGAAC 3′

C: 3′ CAAAACCGTGTAGGACTAATGAT-
TATT**CCTAGG**GCG 5′

Primers N and C were designed to create *Nde*I site *CATATG* at the 5′- and *Bam*HI site *GGATTC* at the 3′-coding ends.

F: 5′ **CATAcG**AGAGTAAGATTTTGC-
CTGGCCCTGATGGT**GGcTCC**AT 3′

R: 3′ **GTATgC**TCTCATTCTAAAACG
GACCGGG- ACTACCA**CCgAGG**TA 5′

Primers F and R were designed to create point mutations at internal *Nde*I and *Bam*HI sites (nucleotides No. 364 and No. 397) which are shown as small letters in the **F** and **R** oligonucleotide sequences. The PCR amplification of the *Llpr10.1a* gene was performed in three separate steps which are shown in Table 1.

Table 1. Steps of PCR amplification of the *Ll*PR10.1A coding DNA sequence

PCR	Template	Primers	Product
No. 1	*Ll*PR10.1A cDNA	N + R	P1
No. 2	*Ll*PR10.1A cDNA	C + F	P2
No. 3	P1 + P2	N + C	P3

The product of the PCR No. 3 (P3) had two point mutations at position No. 364 and No. 397 within the same open reading frame.

The PCR amplifications were performed in 50 µl reaction mixtures containing: 10 mM Tris/HCl buffer, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 20 ng of plasmid DNA carrying *Llpr10.1a* cDNA clone (PCR No. 1 and 2) or 20 ng of P1 and P2, 25 pmoles of each primer (in the single steps shown in Table 1, different pairs of primers were used), 200 mM of each dNTP and 1 U of TaqI DNA polymerase. The reaction was performed in three steps per PCR cycle: denaturation at 94°C for 1

min, annealing at 55°C for 1 min and polymerisation at 72°C for 2 min (30 cycles) using the termocycler Type PTC-200 (MJ Research). Five µl of mixture was analysed by electrophoresis on 1.2% agarose gel in 1 × TAE buffer (0.04 M Tris-acetate, pH 8.5, 0.002 M EDTA). The amplified coding DNA fragment (P3) was then digested with *Nde*I and *Bam*HI and inserted into pET-3a and pET-15b expression plasmids by direct asymmetrical cloning. Transformations were carried out by the $CaCl_2$ method (Mandel & Higa, 1970). All others molecular cloning procedures were carried out as described by Sambrook *et al.* (1989).

Overexpression of the recombinant LlPR10.1A protein in E. coli cells, analytical scale. The BL21(DE3)pLysS cells carrying either pET-3a/*Llpr10.1a* or pET-15b/*Llpr10.1a* plasmid were grown in 3 ml LB-medium supplemented with ampicillin and chloramphenicol until the A_{600} reached a value of 0.8 (3–3.5 h) and then overexpression of the *Llpr10.1a* gene was induced by adding IPTG to a final concentration of 0.5 mM when the pET-3a plasmid was used and 1.0 mM in the case of pET-15b. Then, *E. coli* cells were grown for another 3 and 5 h and collected in Eppendorf tubes at 10000 r.p.m. for 2 min. The cells were lysed in 200 µl 1 × Laemmli sample buffer and analysed by 15% SDS/PAGE (Laemmli, 1970).

Preparative scale production and purification of the recombinant protein. Production of recombinant protein on a preparative scale was carried out in 5 l bioreactor type Bioflo III (New Brunswick Scientific). The bacterial cells were grown until the A_{600} reached a value of 0.8, induced for 5 h after the addition of IPTG and collected in Beckman preparative centrifuge type J2-21 at 6000 r.p.m. for 15 min at 4°C. For lysis of cells the "freeze and thaw" procedure was applied. Finally frozen cells were transferred on ice and resuspended in 40 ml of 50 mM Tris/HCl, pH 8.0, 50 mM EDTA, 10 mM $MgCl_2$ and 3 vol. of 8 M urea, 10 mM $MgCl_2$ were added (to the final urea concentration of 6 M), in order to dissolve the recombinant protein produced in bacterial cells as insoluble "inclusion bodies". After 1 h incubation at 37°C, the lysate was centrifuged at 15 000 r.p.m. for 30 min at 4°C in Beckman preparative centrifuge. The supernatant containing recombinant protein was collected, diluted with 2 vol. of 20 mM Tris/HCl, pH 8.0, 5% glycerol and fractionated by ammonium sulphate precipitation. The fraction 0–80% sat. was taken for further purification. The precipitate was dissolved in 45 ml of 20 mM Tris/HCl, pH 8.0, 0.5 M NaCl, 6 M urea, 5% glycerol and dialysed against this buffer (3 × 0.5 l) for 18 h at 4°C. The dialysed protein preparation was clarified by centrifugation at 15000 r.p.m. for 15 min at 4°C and passed over 50 ml DE 52 cellulose column to separate the protein fraction from bacterial chromosomal DNA contamination.

DE 52 cellulose chromatography of recombinant protein in Na-phosphate buffer, pH 7.5. The protein fraction eluted from the DE 52 cellulose with Tris/HCl buffer, pH 8.0 containing 0.5 M NaCl (purified from bacterial DNA) was dialysed against 20 mM Na-phosphate, pH 7.5, 10% glycerol and submitted to rechromatography on DE 52 cellulose column in Na-phosphate, pH 7.5; fractionation was carried out by stepwise elution with increasing concentration of NaCl from 80 to 480 mM.

Size exclusion chromatography on Superdex 75 HiLoad 16/60 FPLC column. The DE 52 cellulose fractions containing the recombinant *Ll*PR10.1A protein were concentrated with Sephadex G150 (in dialysing tubing) to 1 or 2 ml (up to 12.5 mg/ml) and size fractionated by FPLC on the Superdex 75 HiLoad 16/60 column (up to 25 mg protein fraction per run) with the flow rate of the elution buffer at 0.3 ml/min. Fractions of 1.35 ml were collected. The elution buffer contained 20 mM Na-phosphate, pH 7.5, 100 mM NaCl, 10% glycerol.

Chelating chromatography of $(His)_6$-LlPR10.1A protein on His.Bind Resin. Thrombin cleavage. The recombinant *Ll*PR10.1A protein produced in the pET-15b expression plasmid as a fusion protein was preliminarily fractionated from crude bacterial lysate by ammonium sulphate precipitation and DE 52 cellulose chromatography and subjected to chelating chromatography on Ni^{2+}-charged His.Bind Resin. A column of 2.5 ml was used to separate protein prepara-

5′t tta tac tct ata aca ctc cca cta tct tat tct cat act ttc ctt 46

M G I F A F E N E Q 10

ctg tct cca tta tca atc **ATG** GGT ATT TTT GCT TTC GAA AAT GAA CAA 94

S S T V A P A K L Y K A L T K D 26

TCC TCT ACT GTG GCT CCA GCT AAA CTA TAC AAA GCT CTC ACA AAA GAT 142

S D E I V P K V I E P I Q S V E 42

TCT GAT GAA ATC GTC CCA AAG GTG ATT GAG CCA ATC CAA AGT GTT GAA 190

I V E G N G G P G T I K K I I A 58

ATT GTT GAA GGA AAT GGA GGA CCA GGA ACT ATC AAG AAG ATA ATT GCT 238

I H D G H T S F V L H K L D A I 74

ATT CAT GAT GGT CAC ACT AGC TTT GTG CTG CAC AAA CTT GAT GCA ATA 286

D E A N L T Y N Y S I I G G E G 90

GAT GAA GCT AAC TTG ACA TAC AAC TAC AGC ATA ATT GGA GGT GAA GGG 334

L D E S L E K I S Y E S K I L P 106

TTG GAT GAA AGT TTA GAG AAA ATC *TCA TAT GAG* AGT AAG ATT TTG CCT 382

G P D G G S I G K I N V K F H T 122

GGC CCT GAT GGT *GGA TCC* ATT GGA AAA ATT AAT GTT AAA TTT CAT ACC 430

K G D V L S E T V R D Q A K F K 138

AAA GGT GAT GTG CTA TCA GAA ACT GTG CGT GAT CAA GCA AAA TTC AAG 478

G L G L F K A I E G Y V L A H P 154

GGA CTT GGA CTT TTT AAG GCA ATT GAA GGT TAT GTT TTG GCA CAT CCT 526

D Y * 156

GAT TAC **TAG** att ctc ata aaa aac act act gct act act ttt act ttt 574

ggt tga ttc atg caa ata att atc ata gta agt gtg tgt gtg gtt gtt 622

tgg ttt ttg taa caa gag ccc ttt gat ata tac atg tgg ctc agt tga 670

att gcc ttg tgt gtt tga act ggt gtt tat gtt ttg gca aca ctt gta 718

ttc tgc aat aaa taa tga ata aga ttt gtg ttt gta taa aaa aaa aaa 766

aaa aaa aaa 3′ 775

Figure 1. Full-length cDNA copy coding for *Lupinus luteus* PR10.1A protein.

The nucleotide sequence was registered in EMBL GeneBank under the accession No. X79974. Start codon (ATG) and stop codon (TAG) are in bold face; internal *Nde*I and *Bam*HI recognised sequences are in italics; the 5′- and 3′-noncoding cDNA fragments are in small letters. The putative polyadenylation signal aataaa is underlined.

tion from 0.5 l bacterial culture. After DE 52 cellulose chromatography the protein was dialysed against binding buffer containing 5 mM imidazole, 0.5 M NaCl, 20 mM Tris/HCl, pH 7.9, and applied to the Ni^{2+}-charged resin. The column was washed with the above buffer containig 60 mM imidazole. The fusion protein containing the $(His)_6$ tag was eluted from the column with 1 M imidazole, 0.5 M NaCl (in 20 mM Tris/HCl, pH 7.9). All steps were performed in the presence of 2 M urea. The eluted protein was concentrated in Centricon-10 concentrator and dialysed against buffer containing 20 mM Tris/HCl,

pH 8.4, 150 mM NaCl, 2.5 mM $CaCl_2$ and cleaved with thrombin protease. The reaction was carried out at room temperature for 16 h with 1 U thrombin per 1 mg of purified $(His)_6$-*Ll*PR10.1A protein. After protease cleavage, the reaction mixture was dialysed against binding buffer and purified from 2000 polypeptide by rechromatography on His.Bind Resin. The unbound fraction was dialysed against 20 mM Tris/HCl, pH 8.0, 10% glycerol and concentrated (in Centricon-10) to 10 mg/ml. The yield of *Ll*PR10.1A protein purification by chelating chromatography was 4.2 mg of homogeneous preparation per 1 l of liquid *E. coli* culture.

Immunochemical analysis of the recombinant LlPR10.1A protein. Western blot analysis of the recombinant protein was carried out according to the procedure described by Burnette (1981). The homogeneous protein samples were electroblotted onto Immobilon P membrane using MilliBlot-SDE transfer system according to the Millipore protocol. Protein blot was incubated for 18 h with 100-fold diluted rabbit serum containing anti-*Ll*PR10.1A antibody against native plant protein. All incubation mixtures contained Tris-buffered saline, pH 7.5, 1% bovine serum albumin. The antigen-antibody complexes were visualised by exposure of blots to X-ray film after the reaction with ^{125}I-labelled protein A (4 $\times$ 10^5 c.p.m./ml). Rabbit antibodies were prepared according to the earlier described procedure (Axelsen *et al.*, 1973; Sikorski *et al.*, 1989).

Other methods. Protein content in the analysed fractions was measured as described by Bradford (1976). Electrophoretic analysis of protein fractions: was performed in 15% polyacrylamide gel in the presence of SDS (SDS/PAGE) as described by Laemmli (1970).

RESULTS

PCR amplification, cloning and expression of the *Llpr10.1a* coding DNA sequence in *E. coli*

The pBluescript SK plasmid carrying the entire *Llpr10.1a* cDNA of 775 bp (Fig.1) was taken as a template to amplify the DNA fragment coding for the *Ll*PR10.1A protein. The PCR amplification was performed in three steps as described in Materials and Methods section. Two internal primers F and R were designed to create mutations at *Nde*I and *Bam*HI sites. The other two oligonucleotide primers: N and C containing these two unique restriction sites, *Nde*I and *Bam*HI were used to amplify the *Llpr10.1a* coding DNA sequence in order to allow direct subcloning of the amplified gene into the pET-3a and pET-15b expression vectors. The products of the PCR amplifications are shown in Fig. 2. The PCR product P3 was digested with *Nde*I and *Bam*HI restriction endonucleases and subcloned into pET-3a and pET-15b expression vector. Both pET-3a/*Llpr10.1a* and pET-15b/*Llpr10.1a* constructs were verified by method of Sanger *et al.* (1977) and taken for transformation of *E.*

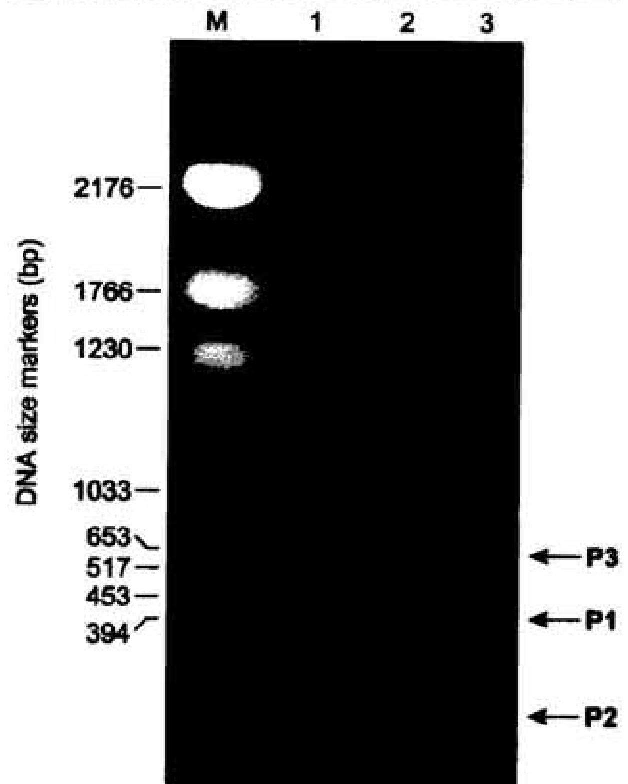

Figure 2. Gel electrophoresis on 1.2% agarose of PCR amplification products of the *L. luteus* PR10.1A coding sequence carrying point mutations at the internal *Nde*I and *Bam*HI sites with preservation of reading frame.

Lane M, DNA size markers; lanes 1–3, products of PCR amplifications: P1, P2 and P3 generated in three single PCR amplifications (for details see Materials and Methods).

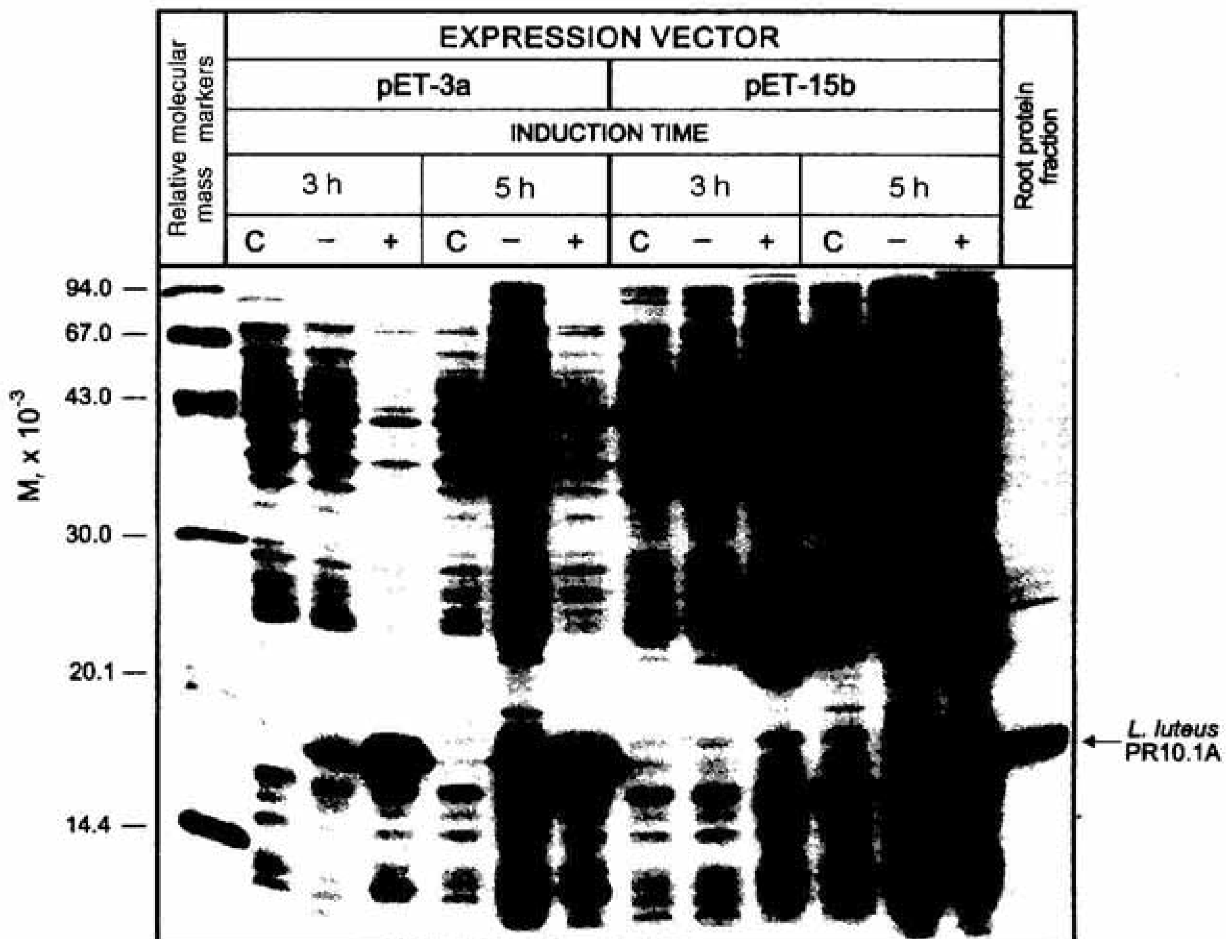

Figure 3. Expression of *L. luteus* PR10.1A cDNA (coding region) in *E. coli* cells using pET-3a and pET-15b expression vectors.

15% SDS/PAGE of bacterial lysates was carried out to analyse the level of recombinant protein synthesis in *E. coli* cells after 3 and 5 h of IPTG-induction: C, proteins of lysates of bacterial cells transformed with pET-3a and pET-15b vector, respectively, which did not carry any coding sequence but were IPTG-induced (as negative controls); −, proteins of bacterial lysates harbouring pET-3a/*pr10.1a* and pET-15b/*pr10.1a* constructs of not induced cells, and +, IPTG-induced cells (3 and 5 h induced cells).

coli strain BL21(DE3)pLysS carrying the IPTG inducible T7 RNA polymerase gene.

The overproduction of the recombinant *Ll*PR10.1A protein was analysed in bacterial lysates by SDS/PAGE (Fig. 3). The recombinant protein overproduced in pET-3a vector migrated in the gel at the position corresponding to that of the native plant protein, whereas the fusion protein overproduced in pET-15b plasmid had a higher molecular mass. This was due to the presence of a fusion peptide (2000 polypeptide carrying the $(His)_6$ tag) at the front of the N-terminus of recombinant protein.

The recombinant protein produced in *E. coli* cells in either pET-3a or pET-15b vector constitutes a significant fraction of the total bacterial protein.

Purification of the recombinant protein from bacterial lysate

The crude bacterial lysates of 5 l cultures were submitted to purification and fractionation according to the flow diagram shown in Fig. 4. The ion exchange chromatography on DE 52 cellulose was used to separate the protein fraction from lipids and bacterial

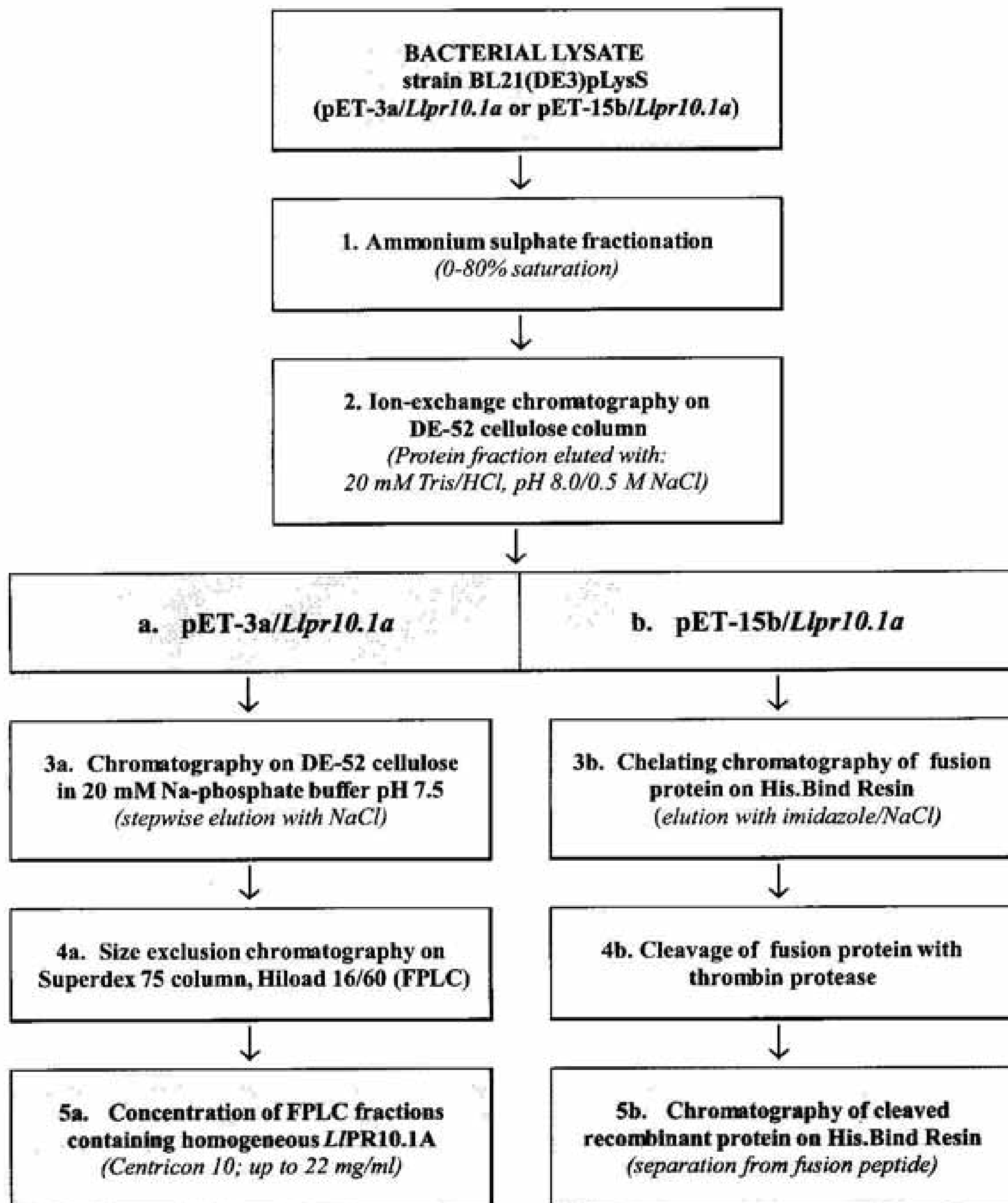

Figure 4. Flow diagram of the purification of the recombinant protein overproduced in *E. coli* cells, strain BL21(DE3)pLysS in pET-3a and pET-15b expression vector.

DNA contaminants. In the preliminary fractionation steps (step 1 and 2, Fig. 4), a substantial enrichment of bacterial lysate in recombinant protein was also achieved. The lysis and DE 52 cellulose chromatography were performed at the presence of 6 M urea in order to solubilise recombinant proteins overproduced in bacterial cells as "inclusion bodies". The dialysis against the buffer containing 10% glycerol allowed to remove urea and renature the recombinant proteins. Further purification of the recombinant *Ll*PR10.1A protein produced in plasmids of pET-series was performed as shown in the flow diagram (Fig. 4). The rechromatography of *Ll*PR10.1A-containing fraction produced in pET-3a/*Llpr10.1a* plasmid on DE 52 cellulose, carried out by stepwise elution with NaCl gradient (cf. Materials and Methods) gave SDS/PAGE pattern shown in Fig. 5.

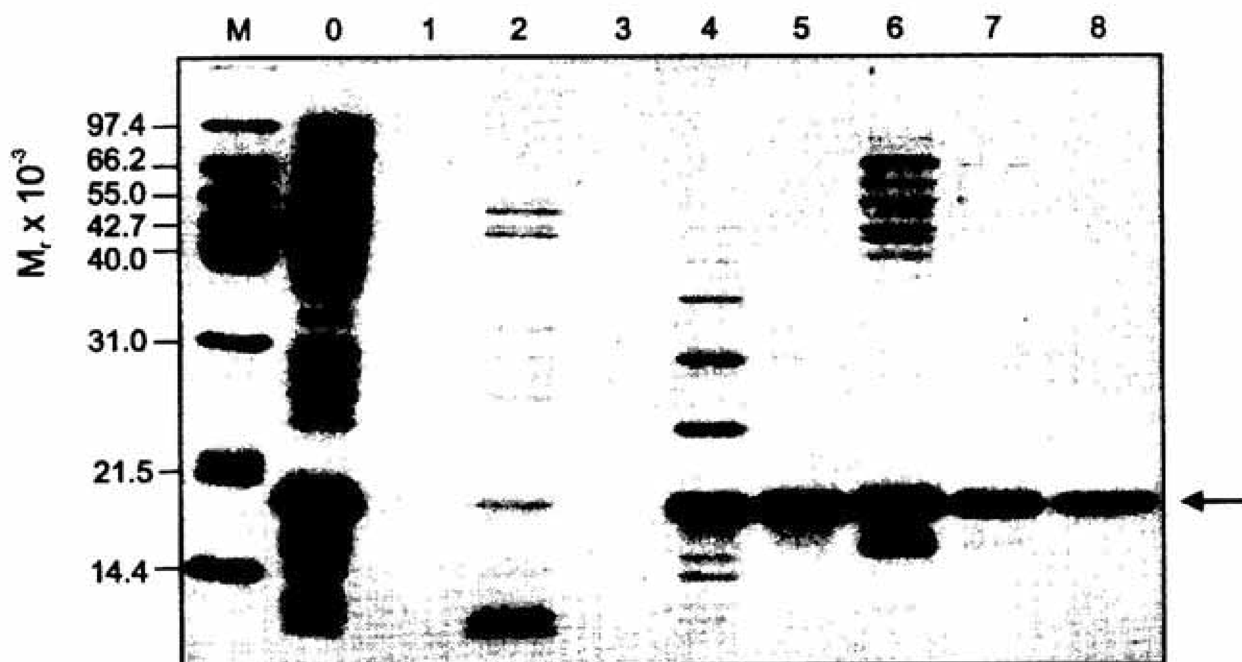

Figure 5. SDS/PAGE analysis of stepwise fractionation of the recombinant *Ll*PR10.1A protein-containing fraction on DE 52 cellulose in the presence of 20 mM Na-phosphate buffer.

The dialysed fraction (205 mg) after DE 52 cellulose chromatography in Tris/HCl buffer, pH 8.0, 0.5 M NaCl was applied to a 20 ml DE 52 cellulose column. The protein was fractionated by stepwise elution with 80–480 mM NaCl in the presence of 20 mM Na-phosphate buffer, pH 7.5. The following buffers were used: 20 mM Na-phosphate and 20 mM Na-phosphate supplemented with NaCl up to 500 mM concentration of Na^+-cations (fractions $F_{1/100}$–$F_{2/500}$ were collected), and 5–8 µg of each eluted fraction was analysed by 15% SDS/PAGE: lane 0, bacterial protein fraction of 0–80% $(NH_4)_2SO_4$ saturation; lane 1, flow through; lane 2, 20 mM wash; lane 3, fraction $F_{1/100}$; lane 4, fraction $F_{2/100}$; lane 5, fraction $F_{1/200}$; lane 6, fraction $F_{2/200}$; lane 7, fraction $F_{1+2/300}$; lane 8, fraction $F_{1+2/500}$. Fractions eluted with 100–500 mM salt were collected as two subfractions, each of 10 ml (20 ml of elution buffer per one step). The subfractions eluted with 300 and 500 mM salt were pooled and analysed as single fractions (lanes 7 and 8).

Fractions eluted between 80 and 480 mM of NaCl were then purified to homogeneity by size exclusion chromatography on Superdex 75 HiLoad 16/60 FPLC column (Fig. 6) and concentrated to 22 mg/ml, using the Centricon-10 concentrator. The yield of recombinant *Ll*PR10.1A protein fractionation and purification was 26.0 mg per 1 l of liquid media.

The recombinant fusion protein produced in pET-15b expression plasmid was partially purified from crude bacterial lysate by ammonium sulphate precipitation followed by DE 52 cellulose chromatography as described for the pET-3a/*Llpr10.1a* construct. The third step was the chelating chromatography on Ni^{2+}-charged His.Bind Resin (Fig. 4, step 3b). The fusion protein was cleaved with thrombin protease and purified from 2000 polypeptide carrying $(His)_6$ tag by rechromatography on His.Bind Resin. Figure 7 shows the results of the 15% SDS/PAGE analysis of successive purification steps of fusion protein, including chromatography on His.Bind Resin before and after cleavage with thrombin protease.

Immunochemical analysis of recombinant protein

Both recombinant protein preparations produced in pET-3a and pET-15b expression vector were analysed by Western blot to prove their identity to the native *Ll*PR10.1A protein. The immunoreaction was performed with rabbit anti-*Ll*PR10.1A antibody produced against native plant protein. The immunoreactivity of both recombinant and native proteins was of the same intensity (Fig. 8).

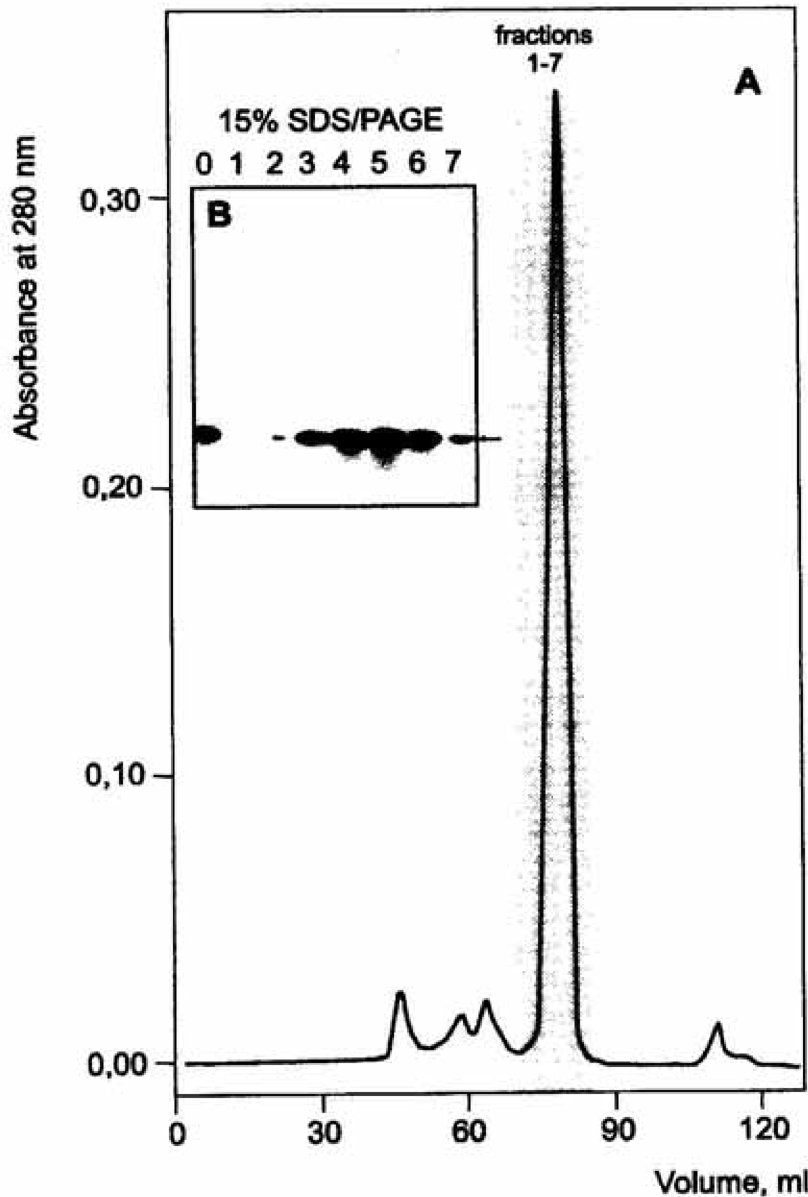

Figure 6. Size exclusion chromatography on Superdex 75 Hiload 16/60 FPLC column of DE 52 cellulose fraction $F_{1/200}$ containing the recombinant *Ll*PR10.1A protein.

Each 1.35 ml fraction was analysed by 15% SDS/PAGE (insert): lane 0, fraction before FPLC separation; lanes 1–7, fractions of the recombinant protein peak.

DISCUSSION

The biochemical and physiological function of plant intracellular pathogenesis-related proteins of PR10 class and their contribution to the defence mechanism still remains unknown. The structural similarity to ginseng ribonuclease suggest their classification as ribonuclease-like PR proteins but there is no evidence for their RNase activity (Moiseyev *et al.*, 1994, 1995; Van Loon *et al.*, 1994). Our studies on yellow lupin proteins led to the identification of two homologous proteins of PR10 class, *Ll*PR10.1A and *Ll*PR10.1B composed of 156 amino-acid residues each (Sikorski *et al.*, 1989; Sikorski, in preparation). The Northern blot hybridization analysis of the transcript level of the two lupin PR10 protein genes clearly demonstrated a high constitutive expression of both lupin homologues in roots of uninfected plants and their down-regulation in mature nodules after inoculation with *Bradyrhizobium* sp. (*Lupinus*). The transcripts of both genes were also detected in mature plant leaves after infiltration with pathogenic bacteria (Sikorski, unpublished). These results imply that both lupin PR10 proteins are involved in a more general plant development processes as well as in the defence mechanism.

The preparation of large quantities of the protein directly from the plant tissue is al-

Figure 7. SDS/PAGE analysis of purification steps of $(His)_6$-*Ll*PR10.1A protein overproduced in pET-15b expression plasmid.

Protein content was analysed by 15% SDS/PAGE: lane 1, bacterial lysate of non transformed *E. coli* cells; lane 2, bacterial lysate of 3 ml analytical culture of transformed *E. coli* cells, IPTG-induced; lane 3, bacterial lysate of 5 l preparative culture (IPTG-induced) after $(NH_4)_2SO_4$ fractionation (0–80% sat.); lane 4, DE 52 cellulose $(His)_6$-*Ll*PR10.1A-containing fraction; lane 5, $(His)_6$-*Ll*PR10.1A protein purified by chelating chromatography on Ni^{2+}-charged His.Bind Resin column (fraction eluted with 1 M imidazole, 0.5 M NaCl); lane 6, *Ll*PR10.1A protein after cleavage with thrombin protease and rechromatography on His.Bind Resin (fraction which does not bind to the Ni^{2+}-charged resin).

ways a crucial step in any functional or structural studies. To solve this problem the bacterial expression system with the vectors of pET-series was used. The protein overproduced in pET-3a vector appeared as a M_r 17000 product which corresponds to native *Ll*PR10.1A protein. However, the fusion protein synthesised by bacterial cells when pET-15b plasmid was used, had a relative molecular mass by 2000 higher. This was due to the presence of a $(His)_6$ tag-containing peptide at the N-terminus of recombinant protein, and allowed us to use one-step purification procedure of the overproduced protein by chromatography on Ni^{2+}-charged His.Bind Resin. The cleavage of the fusion protein by thrombin protease released the recombinant protein with three additional amino-acid residues (Gly-Ser-His) at the N-terminus. This may be somehow inconvenient, since any additional amino acids added to the native protein may affect its solubility at higher concentrations (10–20 mg/ml). Highly concentrated protein preparations are required for crystallisation and some spectroscopic analyses. Unfortunately, the recombinant protein produced in pET-15b vector could not be used for this kind of experiments. Nevertheless, at lower concentrations the protein is soluble in the presence of 5% glycerol and it can be used as an antigen for antibodies production.

The *Ll*PR10.1A protein overexpressed in *E. coli* cells transformed with pET-3a/*Llpr10.1a* plasmid was purified from bacterial lysate to homogeneity by $(NH_4)_2SO_4$ fractionation (80% sat.) and two chromatographic steps on DE 52 cellulose followed by size exclusion chromatography on Superdex 75 HiLoad 16/60 FPLC column and finally concentrated on Centricon-10 up to 22 mg/ml. The last step allowed to separate the recombinant protein preparation from minor contaminants, i.e. polypeptides smaller than 10000. The yield of the recombinant LlPR10.1A protein purification was 26.0 mg of homogeneous preparation per 1 liter of *E. coli* culture.

Immunochemical analysis of the two recombinant *Ll*PR10.1A proteins produced in pET-3a and pET-15b expression plasmids, performed by the Western blot procedure using rabbit serum containing antibodies against native yellow lupin PR10.1A protein showed that both the recombinant and native protein exhibited identical immunological reactivity. This proved the identity of the recombinant and native proteins.

L. luteus PR10.1A protein expressed in pET system purified to homogeneity, renatured and concentrated to 22 mg/ml was then crystallized for X-ray determination of the three dimentional structure. It was also used as an

Figure 8. Western blot analysis of recombinant proteins — *Ll*PR10.1A^R.

Lane 1, *L. luteus* PR10.1A protein purified from plant roots; lane 2, recombinant *Ll*PR10.1A protein purified from BL21(DE3)pLysS lysate after expression in pET-3a vector; lane 3, recombinant *Ll*PR10.1A protein purified from BL21(DE3)pLysS lysate after expression in pET-15b vector; lane 4, bacterial lysate of non-transformed cell. One μg of each purified protein and 10 μg of bacterial lysate of non-transformed cells, as a negative control was separated by 15% SDS/PAGE, electroblotted onto Immobilon-P membrane and the immunoreaction was carried out with rabbit anti-*Ll*PR10.1A^N antibody. The antigen-antibody complexes were visualised by autoradiography after reaction with ^{125}I-labelled *Staphylococcus aureus* protein A (4×10^5 c.p.m./ml).

antigen to make polyclonal rabbit antibodies for immunochemical detection of PR10 protein in developing lupin roots and root nodules.

This work shows that recombinant proteins overproduced in the applied heterologous bacterial system with different expression plasmids can be successfully used for production of substantial quantities of plant proteins for functional and structural studies.

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