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Cloning, sequence analysis and expression in *Escherichia coli* of the cDNA encoding a precursor of peanut agglutinin

Esperanza Rodriguez-Arango[a], Rafael Arango[a], Rivka Adar[a], Gad Galili[b] and Nathan Sharon[a]

Department of [a]Membrane Research and Biophysics and [b]Plant Genetics, The Weizman Institute of Science, Rehovot 76100, Israel

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The cDNA coding for pre-peanut agglutinin (PNA) was isolated from a bacterial expression library. It codes for a polypeptide of 273 amino acids composed of a hydrophobic signal peptide of 23 amino acids and a mature protein of 250 amino acids. The sequence of the latter is identical to that of native PNA, determined very recently by conventional methods, except that it contains 14 additional amino acids at the C-terminus. Bacterial cells harboring a plasmid with the prePNA–cDNA, produced two PNA cross-reacting proteins: one migrated on SDS-PAGE identically with the native lectin (apparent mol. wt. 31 kDa); the other, at 35 kDa, was a β-galactosidase pre-PNA fusion protein. The former protein possessed an N-terminal sequence identical to that of the mature, native PNA, suggesting that it was processed from the 35 kDa prePNA precursor. Only the 31 kDa protein was exported into the bacterial periplasmic space, and had the ability to bind to galactose-Sepharose. The isolated processed protein had the same hemagglutinating activity as the native lectin, when assayed with sialidase-treated human erythrocytes. Like the native lectin, it did not agglutinate the untreated cells, was not inhibited by *N*-acetylgalactosamine, and was inhibited by Galβ1→3GalNAc 30-times more strongly than by galactose.

Expression; cDNA; Recombinant lectin; Leader sequence

1. INTRODUCTION

Peanut agglutinin (PNA), the lectin from seeds of *Arachis hypogaea*, is a carbohydrate-free protein composed of four identical subunits of mol. wt. 27–28 kDa [1,2] or 24–29 kDa [3]. Each subunit has an extended binding site specific for galactosyl end groups, and pronounced preference for the disaccharide Galβ1→GalNAc [1,4–6]. This specificity allows for the use of PNA to detect the T-antigen on erythrocytes [1], and to separate mature and immature thymocytes of mouse [7] and man [8]. A partial sequence of PNA has been reported previously [9] and, while this work was being completed, a sequence of 236 amino acids of the lectin has been established by conventional techniques [10]. As expected, it shows extensive homology with the sequences of other legume lectins.

In this paper we report the isolation and characterisation of a cDNA encoding prePNA and the expression of the recombinant active lectin in *Escherichia coli*, demonstrating that the precursor is processed by the bacteria and that only the processed form binds carbohydrate. Our results provide a basis for studies of the combining site of the lectin, as well as of the factors that control gene expression in plants.

Abbreviations: PBS, phosphate-buffered saline; PNA, peanut agglutinin; pPNA7, Bluescript plasmid containing prePNA-cDNA from clone C7; rPNA, recombinant PNA.

Correspondence address: N. Sharon, Department of Membrane Research and Biophysics, The Weizmann Institute of Science, Rehovot 76100, Israel. Fax: (972) (8) 468 256.

2. MATERIALS AND METHODS

2.1. *Materials*

Seeds of the 'Shulamit' variety of *Arachis hypogaea* were collected during the months of April to June from plants grown at the Faculty of Agriculture (Hebrew University) and on the Weizmann Institute campus. Native PNA from mature dry seeds of the above variety was prepared in our laboratory [1]. Oligo-d(T) cellulose was from Pharmacia. Restriction enzymes and DNA modifying enzymes were from Amersham, Pharmacia and New England Biolabs. Predigested λ Zap II/*Eco*RI cloning kit and picoBlue immunodetection kit were from Stratagene. Rabbit polyclonal anti-PNA antiserum was a gift from Dr. Antonio De Maio. Goat anti-rabbit alkaline phosphatase conjugate was from Biomakor (Rehovot) and *Vibrio cholerae* sialidase was from Behringwerke. DNA sequencing reagents and isopropyl β-D-thiogalactopyranoside were from US Biochemical Corp. Synthetic oligonucleotides were prepared by the Chemical Services of The Weizmann Institute of Science. Sugars were from commercial sources, of the highest purity available.

2.2. *Purification of poly(A)⁺ RNA*

Seeds at mid-maturation (0.3–0.6 g wt. each) were used for the preparation of membrane-bound polysomes, which served for the isolation of polysomal RNA, essentially as previously described [11]. Poly(A)⁺ RNA was obtained by oligo-d(T) cellulose chromatography [12].

2.3. *cDNA cloning*

Double-stranded cDNA was prepared from poly(A)⁺ RNA, using the Amersham cDNA synthesis system. The cDNA products were preprared for cloning using standard procedures [13] and were ligated to the expression vector λ Zap, which is capable of generating fusion

proteins with the N-terminal partion of β-galactosidase [14]. *E. coli* XL1-Blue cells were used as the host strain for amplification and screening of the library with rabbit polyclonal anti-PNA antiserum, using the immunoscreening kit from Stratagene. Positive clones were further characterised by analysis of crude lysates using the anti-PNA antiserum.

2.4. *DNA sequence determination*

DNA sequence analysis was carried out by the dideoxy chain-termination method [15], using Sequenase according to the US Biochemical Corp. protocol. Double-stranded Bluescript plasmid containing prePNA-cDNA was used for sequencing of the coding strand in the forward direction. The rescued strand of the recombinant Bluescript plasmid, which contains the non-coding strand, was used for single strand sequencing in the reverse direction. Analysis of the sequences of both strands if the prePNA–cDNA fragment was completed, using synthetic oligonucleotides as primers, complementary to the coding strand in positions 79–93, 331–347 and 640–655, and to the non-coding strand at positions 899–914, 705–720 and 428–442.

2.5. *Expression of recombinant prePNA–cDNA*

E. coli XL1-Blue cells carrying the recombinant Bluecript plasmids were used for expression of prePNA-cDNA as previously reported [16]. Cultures were induced by the addition of isopropyl β-D-thiogalactopyranoside to 0.4 mM, and incubated at 25 or 37°C. Cells were collected by centrifugation, suspended in 1 ml phosphate buffered saline (PBS; 10 mM KH_2PO_4, 0.04 M Na_2HPO_4, 0.9% NaCl, pH 7.4) and sonicated 10 × 30 s on mark 7 with a Branson B-12 sonicator. The resulting crude lysate was centrifuged at 15,000 × *g* for 15 min at 4°C; both the pellet and the supernatant obtained were analysed by 12% SDS-PAGE and immunoblotting.

To examined whether the lectin is secreted into the periplasmic space, 10 ml cultures of bacteria were grown, induced as above, and collected, followed by osmotic shock. For this purpose the cells were washed with 0.2 vol. of 10 mM Tris-HCl, pH 8, then treated with 0.05 vol. of 20% sucrose in 30 mM Tris-HCl, pH 8, 1 mM EDTA, for 30 min on ice, followed by centrifugation (6,000 × *g*, 10 min). The supernatant was removed and the cells were osmotically shocked by adding 0.05 vol. of cold 0.5 mM $MgCl_2$ for 30 min on ice. The cell residue was removed by centrifugation and the supernatant was analyzed by immunoblotting.

To obtain larger quantities of the recombinant PNA (rPNA), transformed cells carrying the recombinant Bluescript plasmid were grown in 10 l fermentors using the same conditions as for the small scale expression experiment, with induction overnight at 25°C. The cells were then collected, suspended in 100 ml of PBS, sonicated 10 × 1 min and centrifuged at 12,000 × *g* for 30 min.

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1                aaccaatccttatcaacctccaactgcacc atg aaa cca ttt tgt gtt ttt ctt act ttc ttt ctc ttg cta   72
                                               Met Lys Pro Phe Cys Val Phe Leu Thr Phe Phe Leu Leu Leu

73  gca gca agt agt aag aag gtg gac tca gcc gaa aca gtt tcc ttc aac ttc aac tct ttc agc gaa gga aac ccc  147
    Ala Ala Ser Ser Lys Lys Val Asp Ser Ala Glu Thr Val Ser Phe Asn Phe Asn Ser Phe Ser Glu Gly Asn Pro

148 gca ata aat ttc caa ggt gac gtc act gtt ctt tca aat ggc aat ata caa ctc acg aac ctc aac aag gta aat  222
    Ala Ile Asn Phe Gln Gly Asp Val Thr Val Leu Ser Asn Gly Asn Ile Gln Leu Thr Asn Leu Asn Lys Val Asn

223 agc gtc ggc cgt gtt ctt tat gcc atg ccg gtg cgc att tgg agc agt gcc acc ggc aat gtc gcc agc ttc ctc  297
    Ser Val Gly Arg Val Leu Tyr Ala Met Pro Val Arg Ile Trp Ser Ser Ala Thr Gly Asn Val Ala Ser Phe Leu

298 acc tcc ttc tct ttc gag atg aag gat atc aaa gat tat gat cct gcc gac ggt atc atc ttt ttc att gca ccg  372
    Thr Ser Phe Ser Phe Glu Met Lys Asp Ile Lys Asp Tyr Asp Pro Ala Asp Gly Ile Ile Phe Phe Ile Ala Pro

373 gaa gat acg cag att cct gcc ggc agt att ggt ggt gga acc tta ggc gtc tct gac act aaa ggg gcg ggt cac  447
    Glu Asp Thr Gln Ile Pro Ala Gly Ser Ile Gly Gly Gly Thr Leu Gly Val Ser Asp Thr Lys Gly Ala Gly His

448 ttt gtt gga gtg gag ttt gat acc tat tcc aac agt gag tac aac gat cca ccc act gat cac gtt gga att gat  522
    Phe Val Gly Val Glu Phe Asp Thr Tyr Ser Asn Ser Glu Tyr Asn Asp Pro Pro Thr Asp His Val Gly Ile Asp

523 gta aat agc gtg gat tcg gtg aag acc gtg cca tgg aat agt gtg agt gga gca gtg gtg aaa gtg act gtg ata  597
    Val Asn Ser Val Asp Ser Val Lys Thr Val Pro Trp Asn Ser Val Ser Gly Ala Val Val Lys Val Thr Val Ile

598 tat gac tct tca aca aag aca ttg agt gtt gct gtg acc aac gac aat ggc gat att acc acc att gct caa gtt  672
    Tyr Asp Ser Ser Thr Lys Thr Leu Ser Val Ala Val Thr Asn Asp Asn Gly Asp Ile Thr Thr Ile Ala Gln Val

673 gtt gat ttg aag gcg aag ctt ccg gag agg gtc aag ttc ggt ttt tct gcc tcc ggc tcc ctt ggc ggt cgt cag  747
    Val Asp Leu Lys Ala Lys Leu Pro Glu Arg Val Lys Phe Gly Phe Ser Ala Ser Gly Ser Leu Gly Gly Arg Gln

748 ata cat ctc atc cgt tca tgg tct ttc act tca acc ttg ata aca aca acc aga aga agc atc gac aat aac gaa  822
    Ile His Leu Ile Arg Ser Trp Ser Phe Thr Ser Thr Leu Ile Thr Thr Thr Arg Arg Ser Ile Asp Asn Asn Glu

823 aag aaa ata atg aat atg gca agt gca tgatttggttttctacgtacgctacctagtacttttaaagaaagaccagacctttgtgtgttat  912
    Lys Lys Ile Met Asn Met Ala Ser Ala End

913 caacttttgtttctttgtgtttaaattaggttgtatggttcaatatttccactaatcatggtctgacggttgaccgtttaaataaaataacaggtccagt 1011

1012 ttggtaaaaa 1021
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Fig. 1. The nucleotide sequence of the prePNA–cDNA was determined by the dideoxy chain-termination method [15], using a double-stranded plasmid, Bluescript, as sequencing vehicle. The deduced amino acid sequence is below the nucleotide sequence; the leader sequence is underlined. Bold underlined letters in the DNA sequence show the polyadenylation signal.

2.6. *Purification of rPNA by affinity chromatography*

The supernatant (100 ml) of a crude bacterial lysate from a large scale preparation was applied at room temperature to a column (4 ml) of Sepharose-bound galactose [1] at a flow rate of 1 column vol./h. The column was washed with PBS until no material absorbing at 280 nm was detected in the effluent ($OD_{280} < 0.05$). The bound protein was eluted with 0.2 M galactose in PBS (1.5 ml/fraction), pooled, extensively dialyzed against water and concentrated in an Amicon untrafiltration stirred cell with a PM-10 membrane to approximately 0.5 mg/ml, estimated by absorption at 280 nm, $A^{1\%}_{280} = 7.7\ cm^{-1}$ [1].

2.7. *Hemagglutinating activity and its inhibition*

Hemagglutinating activity in the absence or presence of sugars was assayed according to the serial dilution methods in microtiter plates as described, using sialidase-treated human type A erythrocytes [1].

2.8. *N-Terminal sequence determination*

The affinity-purified lectin (40 μg) was run in a 12% SDS-gel and electroblotted onto a polyvinylidene difluoride membrane [17]. The band corresponding to the lectin was cut out from the Coomassie blue-stained membrane and sequenced on a gas-phase Applied Biosystem automatic sequencer, model 475A.

3. RESULTS AND DISCUSSION

Studies on the regulation of expression of lectins in legume seeds (e.g. of *Erythrina corallodendron* [18], *Dolichos biflorus* [19] and soybean [20]), demonstrated that their messages begin to accumulate during mid-maturation of the seeds, coinciding with the period of maximal production of the lectin. We were able to confirm, by Western blot analysis of extracts of peanut seeds, that the lectin starts accumulating at about mid-maturation

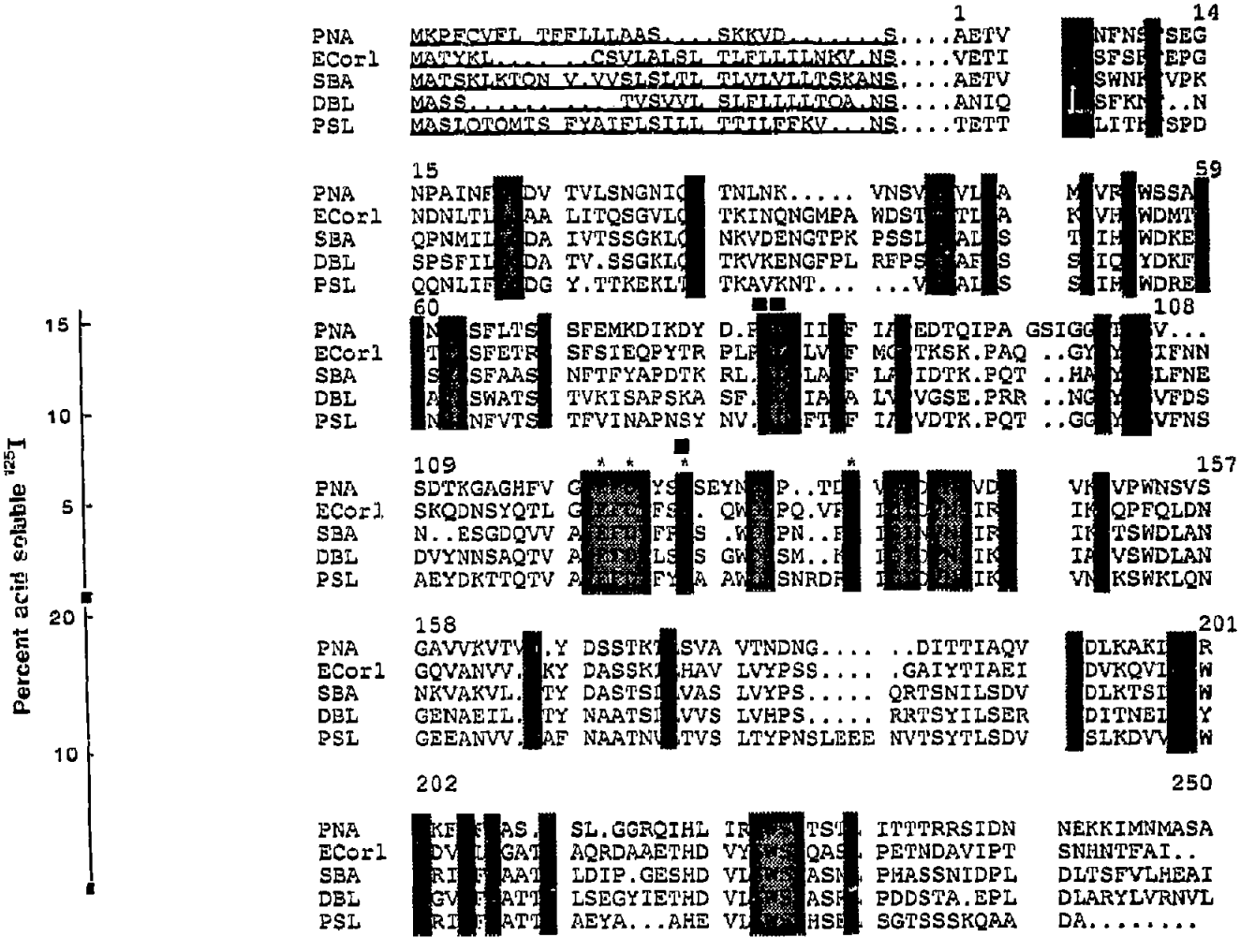

Fig. 2. Comparison of the amino acid sequences of the precursors of several legume lectins specific for galactose (PNA, *E. corallodendron* lectin (ECorL) [18], *Dolichos biflorus* lectin (DBL) [22] and soybean agglutinin (SBA) [23]) and mannose (*Pisum savitum* lectin (PSL) [24]). Leader sequences are underlined. The invariant residues are shadowed. Amino acids involved in metal binding are marked with * [25]; those forming key contacts with the bound sugar (galactose or mannose) are marked with ■ [26]. The numbering of the amino acids is for the sequence of PNA.

(approximately 15 days from the beginning of pod enlargement). In vitro translation of mRNA from seeds at this developmental stage demonstrated that it contained lectin messages; therefore, such seeds were used for the construction of a cDNA library.

3.1. *cDNA cloning*

A cDNA library of 90,000 independent recombinants was constructed in the vector λ Zap [14]. Screening of the amplified library (1.6×10^{11} plaque forming units/ml) with rabbit polyclonal anti-PNA antiserum gave 17 immunopositive clones, the sizes of which varied between 1,000 to 1,200 bp. Cross-hybridization experiments revealed that the clones are highly homologous and probably represent a single species of mRNA.

3.2. *prePNA–cDNA sequencing*

The complete nucleotide sequence of both DNA strands from the insert present in clone C7 (pPNA7 in brief) contains 1,021 nucleotides with a protein-coding region of 819 nucleotides, beginning with an initiation codon, ATG, at positions 31–33, and ending with a stop cocon TGA, at positions 850–852 (Fig. 1). The fragment has a 5′ non-coding region of 30 nucleotides, as well as a 3′ non-coding region of 172 nucleotides before the poly(A)$^+$ tail, containing one polyadenylation site, AATAAA, at positions 992–997. It encodes a pre-protein of 273 amino acids consisting of a leader sequence of 23 amino acids and a polypeptide of 250 amino acids, the latter with a calculated mol. wt of 26,795 Da. The leader is highly hydrophobic, as expected, but differs markedly in size and sequence from that of other legume lectins, in spite of the great similarity in the primary, secondary and tertiary structures of the latter proteins (Fig. 2).

The sequence of the 250 amino acid polypeptide is identical to the recently published, conventionally derived, sequence of PNA [10], except for 14 additional amino acids at the C-terminal end. Recent analysis of the molecular size of mature PNA by mass spectrometry (N.M. Young, private communication) revealed that it consists of a major constituent of 236 amino acids (mol. wt. 25,191 Da) with two minor constituents, one which lacks a single amino acid at the C-terminus and another with two extra residues, with a combined mol. wt. corresponding to Ile^{237} and Asp^{238} of the cDNA-derived sequence. These data, together with our cDNA-derived sequence, strongly suggest that prePNA is proteolytically processed at the C-terminus, in line with several recent reports demonstrating that seed proteins are processed during transport or after deposition in the vacuoles [21].

Comparison of the PNA sequence with those of *E. corallodendron* lectin, soybean agglutinin, and *Dolichos biflorus* lectin (all specific for gatactose and/or *N*-acetylgalactosamine), and pea lectin (specific for mannose and glucose), shows 54 invariant positions (Fig. 2). They include Glu^{121}, Asp^{123}, Asn^{127} and His^{137}, which correspond to residues originally identified in concanavalin A [25] as ligands of the Ca^{2+} and Mn^{2+} required for activity of legume lectins. Similarly, several of the amino acids comprising the hydrophobic cavity of legume lectins, e.g. Phe^{87}, Val^{202}, and Phe^{225} [25], are invariant, as are those involved in carbohydrate binding (Ala^{82}, Asp^{83}, Gly^{104} and Asn^{127}). These results indicate that PNA probably has a similar tertiary structure as well. The conservation of this basic structure among legume lectins suggests an important physiological role in nature, which has yet to be determined.

3.3. *Expression of PNA in* E. coli*: purification and characterisation of rPNA*

Western blot analysis of soluble and insoluble fractions from the bacterial lysates containing pPNA7, induced at 25 or 37°C, demonstrated the presence of two major immunoreactive proteins, one migrating in the region of 35 kDa, and another migrating in the region of 31 kDa. The latter was also present in the periplasmic space fraction (Fig. 3). Other minor cross-reactive proteins probably represent degradation products of the 35 and 31 kDa proteins. The material purified from large scale crude cell lysates by affinity chromatography on immobilized galactose gave, on SDS-PAGE, a single band migrating at a rate close to that of the native lectin, with an apparent mol. wt. of 31 kDa (Fig. 4, lanes A1 and B2), a value higher than the mol. wt. reported in the literature for the PNA subunit, or the value calculated by us (26,795 Da). Typically, 0.3 mg of affinity-purified active rPNA (measured by OD_{280}) was obtained per liter of culture.

The minimal hemagglutinating activity of this purified protein (rPNA), when tested with sialidase-treated human erythrocytes, was 0.25 μg/ml, the same as found with the native lectin; the untreated erythrocytes were not agglutinated by either lectin at 100 μg/ml. Both the recombinant and native lectins are equally susceptible to inhibition by galactose (minimal inhibitory concen-

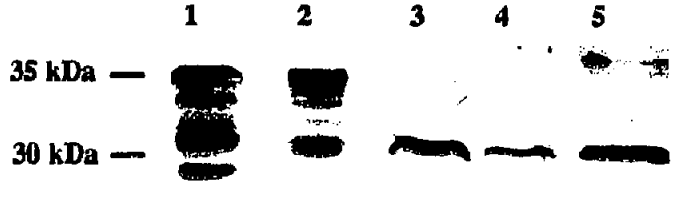

Fig. 3. Western blot analysis of extracts of *E. coli* XL1-Blue cells carrying pPNA7. Crude lysates from bacteria carrying pPNA7 were size fractionated on 12% SDS-PAGE and transferred to nitrocellulose membrane. Blots were probed with polyclonal rabbit anti-PNA antiserum raised against the native lectin. Lanes 1 and 2, XL1-Blue cells with pPNA7 induced at 25 and 37°C, respectively; lanes 3 and 4, fractions from the periplasmic space of XL1-Blue cells induced at 25 and 37°C, respectively; lane 5, native PNA.

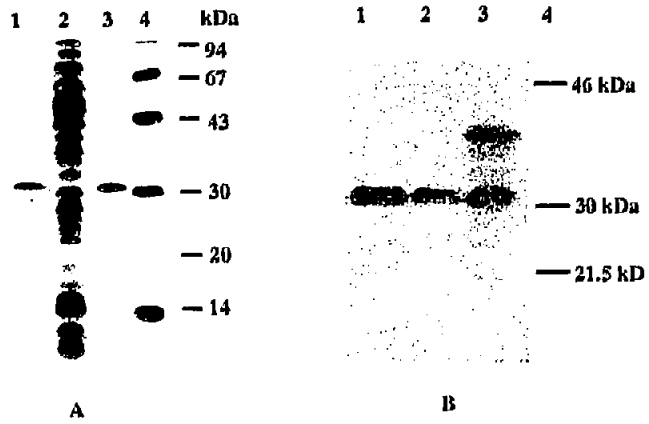

Fig. 4. Isolation of rPNA from *E. coli*. Protein samples were analysed on 12% SDS-PAGE and stained either with Coomassie brilliant blue (A) or transferred to nitrocellulose membrane and probed with polyclonal rabbit anti-PNA antiserum raised against the native lectin (B). Lanes A1 and B2, rPNA purified by galactose-Sepharose affinity chromatography; lanes A2 and B3, cell lysate of XL-1-Blue cells containing pPNA7 induced at 25°C; lanes A3 and B1, native PNA; lane A4, mol. wt. markers in kDa (Pharmacia): phosphorylase *b*, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 20,100; bovine α-lactalbumin, 14,400. Lane B4, Rainbow protein mol. wt. markers in kDa (Amersham); ovalbumin, 46,000; carbonic anhydrase, 30,000; trypsin inhibitor, 21,500.

tration 6 mM), and particularly by Galβ1→3GalNAc and its glycosides (Galβ1→3GalNAcα-O-*o*-nitrophenyl and Galβ1→3GalNAcα-O$(CH_2)_8COOCH_3$), which are about 30-times more inhibitory than galactose. Mannose and *N*-acetylgalactosamine at 200 mM were not inhibitory for either lectin. The hemagglutination studies prove that rPNA is functionally equivalent to native PNA.

A single N-terminal sequence, Ala-Glu-Thr-Val-Ser-Phe-Asn-Phe-Asn-Ser-Phe-Ser, was found in rPNA, identical to that of the native lectin [10].

The finding that the 35 kDa band present in crude lysates was not detected in the affinity purified material, either in Coomassie Blue staining or by immunoblotting, suggests that the 31 kDa lectin, but not the 35 kDa form, is active.

The 35 kDa protein probably represents a fusion protein consisting of the pre-lectin (273 amino acids, calculated mol. wt. 29,368 Da) and of a β-galactosidase portion of ~5.1 kDa. The 31 kDa protein represents the mature protein, as is shown by the N-terminal sequence. These data suggest that the pre-lectin is processed into a mature form in the *E. coli* system. It has indeed been demonstrated that eukaryotic signal peptides can be recognized in prokaryotes [27].

This is the first report clearly demonstrating that the leader sequence of a plant (legume) lectin can be removed correctly by *E. coli*. Several lectins have been expressed in *E. coli* [16,28–30] but their cDNAs were usually devoid of the regions coding for the signal peptide.

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