Volume 250, number 2, 157–160 FEB 07253 July 1989

Lectin-like proteins accumulate as fragmentation products in bean seed protein bodies

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Received 10 April 1989

The gene sequence of the *Phaseolus vulgaris* L. seed lectin-like protein (LLP) and its precursor (~ 40 kDa) which is associated with the endoplasmic reticulum has been described, while the molecular characteristics of the mature protein and its cellular localization are still unknown. Our data, obtained using antibodies raised against a fusion protein, which was produced in *Escherichia coli* and contained most of the LLP sequence, indicate that mature LLP accumulates in the protein bodies of cotyledon cells. LLP consists of several polypeptides in the M_r range 15000 to 20000, which are a result of proteolytic fragmentation of the protein precursor.

Fusion protein; Lectin; Seed protein; (*Phaseolus vulgaris*)

1. INTRODUCTION

Two lectins accumulate at high levels in the seeds of the common bean (*Phaseolus vulgaris* L.). These are phytohemagglutinin (PHA) and the related arcelin [1,2]. A cDNA, and the corresponding gene, coding for a lectin-like protein (LLP) have also been characterized [3,4]. It has been proposed that the genes coding for these proteins arose by divergence and duplication of a common ancestral gene [5]. This evolution could be related to some advantage against insect predators, both arcelin and PHA being related to pest resistance [5,6]. This suggests that LLP might also play a similar defense role in the seed. To prove this hypothesis, it is necessary to identify LLP in bean seed extract and to purify it.

In vitro translation of mRNA hybrid-selected using an LLP cDNA clone yielded a polypeptide of M_r ~28000 [7]. The cDNA sequence codes for a 244 amino acid polypeptide comprising a 21 amino acid putative signal peptide and five potential *N*-glycosylation sites [3]. We have recently shown that, in the Greensleeves cultivar, newly-synthesized LLP is an abundant glycopolypeptide of M_r ~40000, with probably four *N*-linked oligosaccharide side chains, and that it is associated with the endoplasmic reticulum (ER) of developing cotyledon cells [8]. None of the abundant polypeptides that were related to the LLP precursor have been identified so far in the seed, indicating that the protein either does not accumulate or is processed to a mature form with markedly different electrophoretic mobility, as happens to other seed proteins [9].

To discriminate between these two possibilities we have raised antibodies against a fusion protein produced in *E. coli*, that contains most of the LLP amino acid sequence, and we have used these antibodies to challenge bean seed extracts.

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2. MATERIALS AND METHODS

An 800 bp *Cla*I fragment from plasmid pB7LLP [10], containing the sequence coding for LLP amino acids 9–223, was end-filled and subcloned into the end-filled *Bam*HI site of the expression vector pET-3a [11]. The ligation mixture was used to transform BL21(DE3) cells [12] and ampicillin-resistant colonies were used to grow small overnight cultures. An aliquot of each culture was used to further inoculate 10 ml of liquid medium and expression was induced as described [12]. Bacteria were collected by centrifugation, lysed in SDS-PAGE sample

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00145793/89/$3.50

buffer, and proteins were analyzed by SDS-PAGE [13]. Several clones expressing high levels of a 28 kDa protein were identified and one (pETLLPa1) was employed for antigen purification. Bacteria from 250 ml of induced culture were collected by centrifugation, resuspended in lysis buffer (50 mM Tris-Cl, pH 7.4, 1 mg/ml lysozyme) and incubated for 30 min at 4°C. The suspension was then frozen and thawed 3 times before being centrifuged for 1 h at 41000 rpm in a Beckman TI 70.1 rotor, at 4°C. The pellet, containing most of the fusion protein, was resuspended in sample buffer and an aliquot was subjected to SDS-PAGE. The gel was stained with aqueous Coomassie (1 vol. of 3% Coomassie brilliant blue in methanol:4 vols of 0.1 M Tris-Cl, pH 7.4) and the 28 kDa major band was excised. The gel slice was ground in a mortar and then incubated overnight at 4°C in elution buffer (50 mM Tris-Cl, pH 7.9, 0.1 mM EDTA, 1 mM DTT, 0.1 M NaCl, 0.1% SDS). The protein was recovered by precipitation with acetone and resuspended in elution buffer. Production of immune sera against the fusion protein (anti-LLP) and against PHA, purified from bean seed extract [14], was as described in [13].

Purification of polysomal RNA from developing bean (cv Greensleeves) cotyledons, construction of the plasmid pB7LLP and pB7lec1, in vitro transcription and translation were performed as in [10]. When indicated, the in vitro translation system was supplemented with canine pancreatic microsomal membranes, according to manufacturer's specifications (Amersham, Bucks, England). ER-enriched subcellular fraction and protein bodies (PB) were isolated from developing cotyledons as described [15]. Total protein was extracted from mature seeds by homogenization in 20 mM Na borate, pH 9 (10 ml/g tissue), and proteins were recovered in the supernatant after 20 min centrifugation at 20000 × *g*. SDS-PAGE and fluorography were as in [13]. Immunoprecipitation was according to [16], using the anti-LLP serum at a final dilution of 1:200. After SDS-PAGE, proteins were blotted on nitrocellulose sheets (S&S BA 85, 0.45 μm) and Western blot developed according to [17], using sera at a 1:500 dilution and peroxidase-linked anti-rabbit IgG (Miles) as the second antibody. When appropriate, 0.8 ml anti-LLP serum (diluted 1:16) were incubated overnight with about 400 μg of PHA (purified on a thyroglobulin-Sepharose affinity resin) [14], either untreated or made 0.5% in SDS and denatured 5 min at 90°C.

3. RESULTS AND DISCUSSION

Antibodies were raised in rabbit against an *E. coli*-synthesized fusion protein which contained most of the LLP sequence. To investigate whether these antibodies were able to crossreact against LLP, the plasmid pB7LLP, which contains the complete LLP sequence placed downstream of a T7 promoter, was used for in vitro transcription and the synthetic mRNA was translated in the reticulocyte system supplemented with labeled leucine. The products were then challenged with the immune serum. As controls for antibody specificity we used products of in vitro translation of both RNA and synthetic mRNA (obtained by in vitro transcription of plasmid pB7lec1), of bean cotyledons, coding for the LLP-related E-PHA polypeptide. In fig.1, products selected by the immune serum (lanes 8–12) are compared with the same amount of starting material from which each selection was performed (lanes 2–6). Among the total products of bean RNA (lane 6), LLP (asterisk) and PHA subunits (E and L) have already been identified [7,8]. Synthetic LLP mRNA directed the synthesis of a major polypeptide having the expected M_r (lane 4, M_r 28500) which was recognized by the antibodies (lane 10). When microsomal membranes were added to the in vitro translation system, to allow for signal peptide removal and glycosylation [18], in addition to unglycosylated LLP, three slower migrating LLP glycopolypeptides were immunoselected (lane 11, dots). Both glycosylated and unglycosylated forms of LLP were almost quantitatively selected by the antibodies (cf. lanes 10 and 11 with lanes 4 and 5). The two fastest migrating immunoselected polypeptides in lanes 10 and 11 have not been investigated further but probably represent precociously terminated LLP. The related polypeptide E-PHA was weakly recognized when unglycosylated (cf. lanes 2 and 8), while its abundant glycosylated form, synthesized when membranes were added to the translation system, was barely detectable among the immunoselected material (arrowheads in lanes 3 and 9). The mobility of glycosylated E-PHA indicates that it has two oligosaccharide side chains, as bean-synthesized PHA. Immunoselection of in vitro translation products of cotyledon mRNA confirmed that LLP was almost quantitatively selected (cf. lanes 6 and 12), and showed that L-PHA crossreacted with the antibodies more efficiently than E-PHA, although at levels much lower than LLP. The result shown in lane 12 also indicates that there was no crossreaction between the immune serum and the two polypeptides of the major storage protein phaseolin (open circles in lane 6).

Having determined that our anti-serum crossreacted with in vitro synthesized LLP, we looked for the presence of immunologically related polypeptides in bean seeds. Total protein contained in the protein bodies (PB) and ER-enriched preparations from developing cotyledons, and in a total protein extract from mature seeds, was

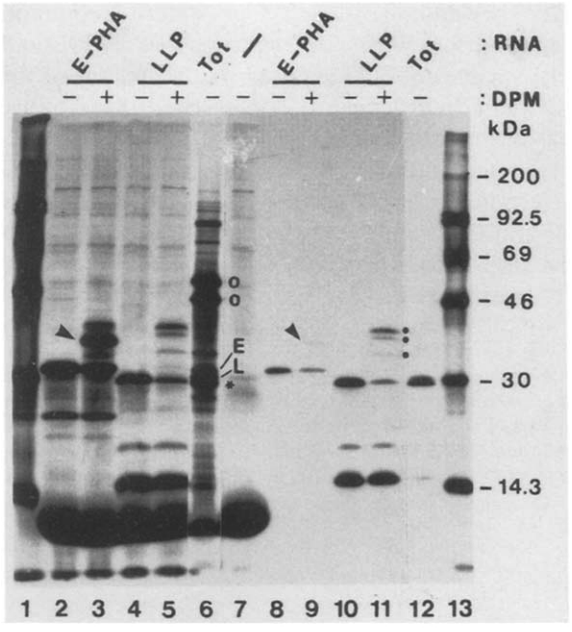

Fig.1. SDS-PAGE and fluorography of in vitro synthesized LLP and PHA. Reticulocyte lysate was supplemented with [^{3}H]leucine and: E-PHA in vitro transcript (E-PHA), LLP in vitro transcript (LLP), bean cotyledon RNA (Tot), no RNA (−). DPM: with (+) or without (−) microsomal membranes added. Lanes 2–7, total products. Lanes 8–12, material immunoselected with the anti-LLP serum. Lanes 1 and 13, molecular mass markers. Lanes 6, 12 and 13 were from a shorter exposure of the SDS-PAGE.

separated by SDS-PAGE and subjected to Western blot analysis. An anti-PHA serum, used as a control, recognized PHA purified on a thyroglobulin-Sepharose affinity column (fig.2, lane 1) [14], as well as PHA contained in the PB extract or accumulated in the mature seed (lanes 3 and 2, respectively). PHA precursor associated with the ER, which has a lower electrophoretic mobility compared to mature PHA [14], could also be detected (lane 4).

The anti-LLP serum recognized five polypeptides from the ER preparation (lane 6). Among these components, two can be purified on a thyroglobulin affinity column and represent ER-associated PHA (lane 5). The other three polypeptides, which have electrophoretic mobilities identical with glycosylated LLP polypeptides synthesized in vitro (fig.1, lane 11 dots), must represent the newly synthesized glycosylated LLP precursor. The presence of three polypeptides in vivo, as well as in vitro, indicates that LLP can have different levels of glycosylation, as is the case with phaseolin polypeptides [13]. The slowest migrating glycosylated polypeptide, which corresponds to the 40 kDa LLP glycopolypeptide we have previously identified, has, most probably, four oligosaccharide side chains [8]. The relative mobility of the other two glycosylated polypep-

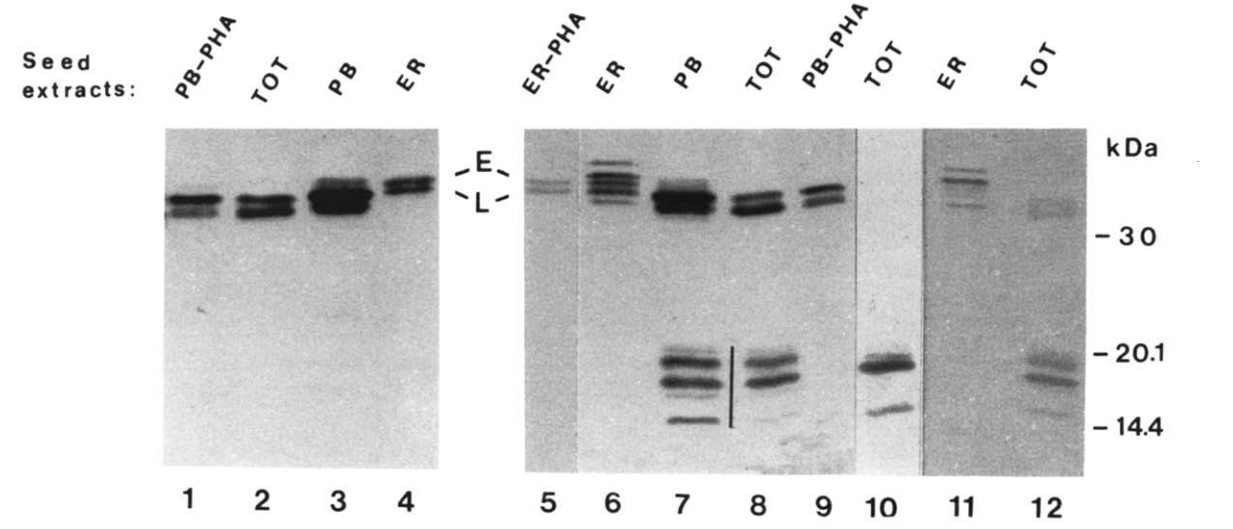

Fig.2. Western blot analysis of seed proteins. Proteins contained in: total protein extract from mature seeds (TOT) and either protein bodies (PB) or ER-enriched (ER) preparations from developing cotyledons were loaded on the SDS-PAGE. Affinity purified PHA was from mature seeds (PB-PHA) and from ER-enriched preparation of developing cotyledons (ER-PHA). Bean cultivars were: Pinto UI 111 (lane 10) and Greensleeves (other lanes). Immune sera were: anti-PHA (lanes 1–4), anti-LLP (lanes 5–10), anti-LLP after incubation with denatured PHA (lanes 11 and 12). Position of molecular mass markers is given on the right.

tides, also with respect to PHA, suggests the presence of three and two side chains. Having entered the secretory pathway of the cell, the LLP precursor could then be transported to the storage compartment, the PB, where the homologous proteins PHA and arcelin accumulate. Among the proteins present in a PB preparation, anti-LLP serum selected, in addition to mature PHA, at least five polypeptides, with molecular masses in the range of 15 to 20 kDa (fig.2, lane 7, vertical bar). This indicates that LLP leaves the ER and is transported into the PB and that, during transport or after arrival to the PB, LLP undergoes further post-translational processing, as is the case for other lectins and storage proteins [9]. The ratio between LLP and PHA in the PB of developing cotyledons (lane 7) is similar to that found in the mature seed (cf. with lane 8). LLP therefore is a protein that accumulates during seed development. The results shown above were confirmed by analysis of a total protein extract from mature seeds of the Pinto UI 111 cultivar, a cultivar lacking PHA [19]. Developing cotyledons of this cultivar contain LLP mRNA and the ER-associated protein precursor at a level comparable to the one determined in the Greensleeves cultivar [7,8]. Analysis of the Pinto UI 111 seed extract showed that LLP accumulates also in this cultivar (lane 10), and evidenced the presence of electrophoretic polymorphism between mature LLP of the two cultivars, a common feature of seed proteins [9]. As expected, no crossreactive material was present in the PHA region of the gel (lane 10). Finally, it is of interest to note that the anti-LLP serum used strongly recognized glycosylated PHA when denatured and blotted (fig.2), while it reacted to a lesser extent with glycosylated PHA in native conditions (fig.1, arrowheads in lanes 3 and 9). The hypothesis that the difference in reaction could be due to denaturation was verified by the following experiment. The anti-LLP serum was incubated with an excess of PHA, either native or denatured for 5 min at 90°C, and then used for Western blot analysis. Pre-incubation with native PHA did not affect reaction on the blot (the result was identical to lanes 6 and 8 in fig.2), while denatured PHA blocked to a great extent detection of PHA on the blot, affecting only slightly detection of LLP (lanes 11 and 12). This would suggest that the observed crossreactivity is mainly due to the presence of one or more common denaturation-dependent epitopes on PHA and LLP, while epitopes exposed on the surface of the native proteins are less conserved. Furthermore, this partial crossreactivity strengthens the idea of a common origin for LLP, PHA and arcelin. The determination of the molecular characteristics of mature LLP and its presence as a stable protein in the seed opens the way to its purification and investigation of its function.

Acknowledgements: We wish to thank L.M. Hoffman (Agrigenetics Corporation, Madison, WI) for providing the pPVL134 and pdlec1 recombinant plasmids and F.W. Studier (Brookhaven Natl. Lab., Upton, NY) for the expression vector pET-3a and bacterial strain BL21(DE3). We also wish to thank M.G. Daminati for technical assistance.

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