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CROSS-REACTION WITH THE PEA LECTIN OF A PROTEIN FROM PROTEIN BODY MEMBRANES

MATHIAS WENZEL, HEINRICH GERS-BARLAG and HAROLD RÜDIGER

Institut für Pharmazie und Lebensmittelchemie der Universität, Am Hubland, D 97074 Würzburg, Germany

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Key Word Index—*Pisum sativum*; Leguminosae; protein bodies; membranes; lectins; cross-reaction.

Abstract—Protein bodies from pea cotyledons contain proteins tightly bound to the membrane with subunit M_rs of 26 000/27 000 and 82 000. The double band of M_r 26 000/27 000 though being clearly distinct from the lectin and its precursor by solubility, cross-reacts with an antibody directed against the pea lectin β-subunit.

INTRODUCTION

In parenchyma cells of mature Leguminosae seeds, protein bodies represent the organelles where storage proteins and lectins accumulate. As in many other cell organelles, protein bodies are surrounded by a single membrane. In a search for possible interactions between protein body constituents and lectins which may point to a biological function of lectins, we studied protein body membranes.

RESULTS AND DISCUSSION

Identification of protein bodies

Protein bodies were isolated from pea cotyledons by centrifugation in a sucrose density gradient as described in Experimental. Under the light microscope, they were found to be spherical in shape and rather uniform in size with diameters of 2.5 ± 0.8 μm ($n = 770$). Cell debri were absent. Treatment with iodine–glycerol led only to weak yellow staining which shows that the preparation was free from starch. No staining was observed with Coomassie Blue unless the protein bodies were lysed (e.g. by 0.05 M Tris buffer pH 8) which shows that the membranes were intact. On electrophoresis of the protein body lysate, the storage proteins, convicilin (M_r of 71.000), vicilin (main bands at M_r *ca* 50 000) and legumin (group at M_r *ca* 40 000 and 20 000, Fig. 1, lane 1) are seen. The lectin and the α-mannosidase occur exclusively in the protein bodies [1, 2] and hence can serve as marker proteins to calculate the protein body yield. Both proteins were determined in extracts prepared from a defined portion of cotyledon flour and alternatively from the protein bodies prepared from the same amount of flour. Protein body yields were 12.0 and 14.4%, and enrichment factors for the marker proteins 1.73 and 1.75, respectively. The same figures were obtained with the β-*N*-acetylhexosaminidase which consequently is also a constituent of protein bodies. Of the α- and β-galactosidase activities, only a part (30–40%) occurs in the protein bodies.

It was not possible to improve the protein body yield. More vigorous homogenization of the cotyledons not only disrupted more cells but also more protein bodies; less intense homogenization left more cells intact.

Preparation and characterization of protein body membranes

In order to obtain the protein body membranes, the protein bodies were lysed in a hypotonic buffer. This has to take place at slightly alkaline pH and at high ionic strength in order to keep the storage proteins dissolved. This and further treatment with guanidinium chloride left only 5% of the initial protein in the membranes. No significant further amount of protein was removed on prolonged washing. Treatment with 10 mM NaOH, however, as used by Mäder and Chrispeels [3] for the purification of the *Phaseolus vulgaris* protein body membranes, removed all proteins from the pea protein body membranes within a few minutes.

The pea protein body membranes were devoid of storage proteins and of α-mannosidase and other glycosidase activities which shows that they are not contaminated by matrix constituents. NADH–cytochrome *c*–oxidoreductase which is a marker enzyme of the endoplasmatic reticulum [4] was also absent.

Purified protein body membranes stain with both Coomassie Blue and Sudan Red. On centrifugation in a sucrose density gradient, they focus at the 40/50% sucrose borderline, i.e. at a density of 1.18–1.23 g ml^{-1}.

Lipid extracts of the membranes were subjected to TLC. Phosphatidylcholine and -ethanolamine but not -inositol could be detected (not shown). The highly purified membranes contain 8–9 μg phosphorus per mg

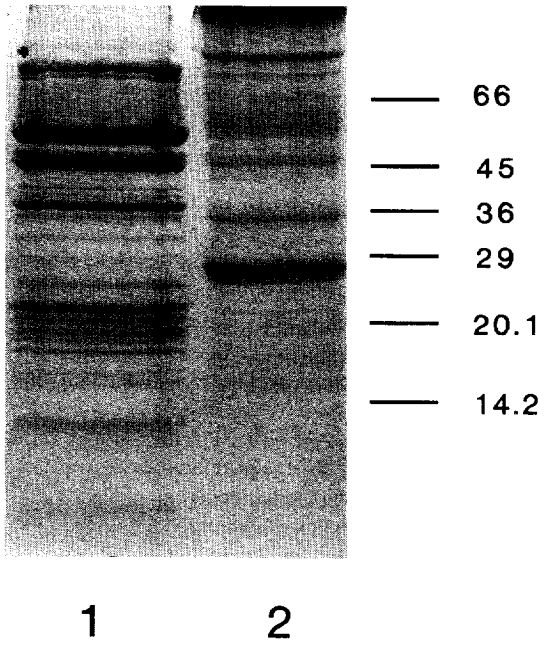

Fig. 1. SDS–PAGE of a pea protein body lysate (lane 1) and of purified protein body membranes (lane 2).

protein. If for membrane lipids an average M_r of 750–800 is assumed, the protein/lipid ratio was about 4:1.

Membrane proteins

The electrophoresis patterns of a protein body lysate (lane 1) and of the purified protein body membrane (lane 2) are compared in Fig. 1. Prominent bands in lane 1 are the storage proteins. These bands have virtually disappeared from the purified membrane in lane 2. Instead, as prominent bands which are firmly bound to the membrane and resist the washing procedure, a doublet at M_r 26 000/27 000 and a single band at M_r 82 000 are seen. These bands are scarcely recognized in the original protein body lysate (lane 1). The double band at M_r 26 000/27 000 is not well resolved in all runs. The bands shown in lane 2 cannot be washed from the membrane unless very drastic means are used (e.g. 10 mM NaOH).

We tested the membrane proteins for possible glycosylation. By the method of ref. [5], no carbohydrate could be detected. After reaction with dansylhydrazine according to ref. [6], a weak fluorescence appeared at M_r 26 000/27 000. This method, however, results also in weak staining of non-glycosylated proteins like the pea lectin and the storage protein legumin. We, therefore, conclude that the membrane protein at M_r 26 000/27 000 is not glycosylated. A possible glycoprotein nature of the protein at M_r 82 000 could not be shown because of a heavily fluorescent smear emerging from the origin (see also Fig. 1, lane 2) which blurred the whole high-M_r region.

The pea lectin consists of the M_r 5800 α- and the M_r 19 800 β-subunits and is always accompanied by small amounts of the M_r 26 800 precursor [1]. We isolated the pea lectin precursor, the α-subunit and the M_r 26 000/27 000 membrane protein by micropreparative electrophoresis and subjected them to a second electrophoresis together with the complete pea lectin, and stained the gel with Coomassie Blue (Fig. 2A). A gel from a parallel run was blotted to nitro-cellulose and the blot incubated with anti-lectin antibody and immunostained as described in Experimental.

In Fig. 2A, lanes 1 to 3, it is seen that the M_r 26 000/27 000 membrane protein appears precisely at the same position as the lectin precursor whereas the α- and β-subunits of the lectin (lanes 2 and 4) are clearly distinct from it. Figure 2B shows that the anti-lectin antibody reacts with both the lectin β-subunit and the precursor molecule (lane 2) whereas it does not recognize the α-subunit (lanes 2 and 4). Remarkably, the precursor reacts relatively more strongly in the immuno than in the Coomassie stain (compare lanes 2 in Fig. 2A and 2B). Lane 1 in Fig. 2B shows that the M_r 26 000/27 000 membrane protein reacts also with the anti-lectin antibody. The high resolution achieved in electrophoresis and Coomassie staining (Fig. 1), however, is lost after blotting and immunostaining. We, therefore, cannot decide which band of the M_r 26 000/27 000 doublet is cross-reactive. Expectedly, the isolated lectin precursor also reacts (lane 3).

Since neither the lectin nor the M_r 26 000/27 000 membrane protein contains carbohydrate, this cross-reactivity cannot be a result of similar glycosylation which in many instances has been shown to lead to cross-reactions among otherwise unrelated plant proteins [7]. Proteins in the M_r 20 000/25 000 and M_r 80 000/100 000 ranges which are tightly bound to protein body membranes have been observed also in other members of the Leguminosae [8, 9], Euphorbiaceae [10] and Cucurbitaceae [11].

In 1979, Pusztai *et al.* prepared protein body membranes from *Phaseolus vulgaris* cotyledons and characterized their protein constituents [9]. Among others, they found proteins of M_r 25 000, 30 000 and 87 000 which they regarded as integral membrane proteins. After isolation, the M_r 30 000 protein agglutinated red blood cells as effectively as the lectin from this plant and reacted with anti-lectin antibodies.

In 1984, Mäder and Chrispeels isolated the M_r 25 000 membrane protein from *Phaseolus vulgaris* protein body membrane by preparative electrophoresis and raised antibodies against it [3]. Interestingly, this antibody not only reacted with its antigen but also with the *Phaseolus vulgaris* lectin (M_r slightly above 30 000). In the reverse experiment, i.e. by using an anti-lectin antibody, they observed no reaction. The authors explained this unexpected unidirectional cross-reactivity by a contamination of the M_r 25 000 preparation used as an antigen with lectin or some lectin-related protein. Though they later showed that the M_r 25 000 membrane protein and the lectin are clearly different [12], the assumed lectin-related material was not studied further.

To our knowledge no detailed information exists about the proteins in pea protein body membranes. Since in pea only one gene codes for the lectin [13], it is unlikely that

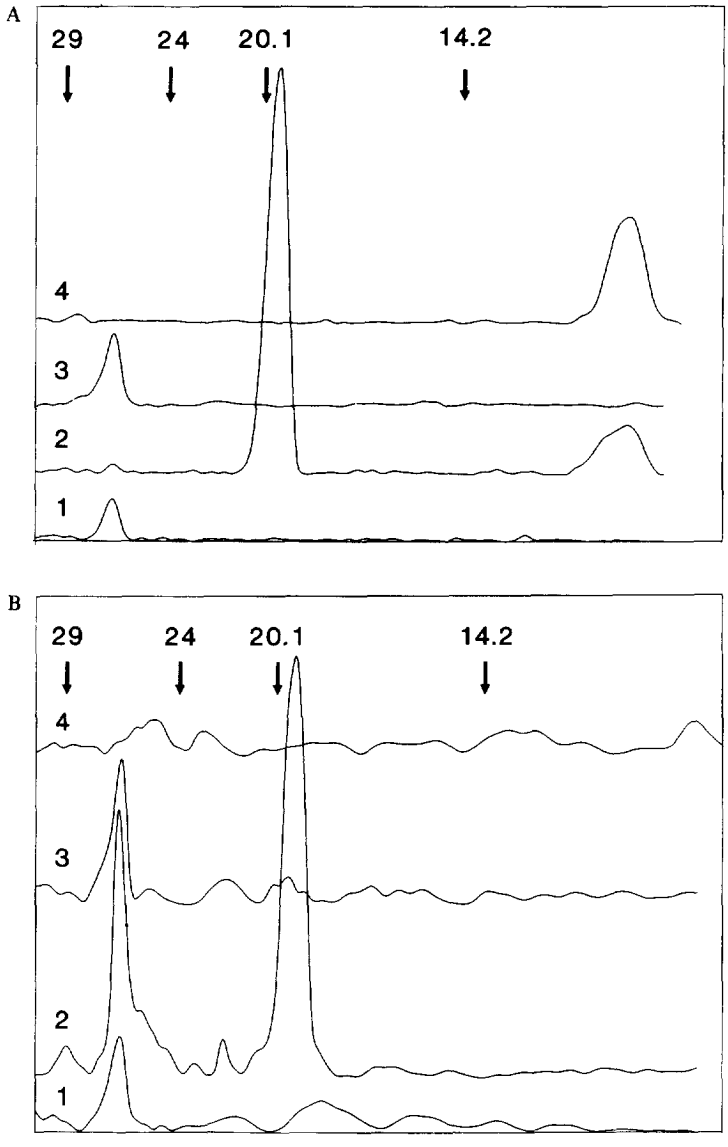

Fig. 2. SDS–PAGE of the M_r 26000/27000 membrane protein (lanes 1), complete pea lectin (lanes 2), precursor of pea lectin (lanes 3) and the pea lectin α-subunit (lanes 4). (A) Gel stained with Coomassie Blue; (B) gel blotted to nitro-cellulose and immunostained as described in the Experimental.

hydrophobic lectin isoforms exist on the gene level which may have remained undetected. Possibly, the membrane protein described in the present paper represents a lectin that has been hydrophobically modified [14–16] after translation.

EXPERIMENTAL

Pea seeds ('Kleine Rheinländerin') were from a local supplier, sucrose from Riedel-de Haën (Seelze) and Merck (Darmstadt), nitro-cellulose membranes BA85 from Schleicher & Schuell (Dassel) and immunochemicals from Sigma (Deisenhofen). All other reagents were of the highest purity commercially available.

Protein bodies. Dry pea seeds were peeled and the embryos removed. The remaining cotyledons were ground to a fine powder. Cotyledon powder (25 g) was homogenized in 180 ml 50% sucrose in H_2O in a Potter-Elvehjem homogenizer (Braun, Melsungen). Undisrupted cells, cell debri and starch were spun down (5 min at 1500 *g*) in a swinging bucket rotor (Beckman, Type JS-13.1). From the supernatant, the protein bodies were sedimented by centrifugation at 10000 *g* for 30 min. The pellet was collected and resuspended in 16 ml 50% sucrose, divided into four portions, placed into four tubes on top of gradients consisting of 10 ml 70%, 4 ml 60% and 5 ml 55% sucrose each, and centrifuged for 1 hr at 19000 *g*. Protein bodies accumulated on top of the 60% sucrose layer. They were collected and stored in 60% sucrose at 4°.

Protein body membranes. For the isolation of membranes, a protein body suspension was added to the

10-fold amount of 0.05 M Tris–HCl, 1 M NaCl, pH 8 at room temp. under vigorous stirring. Under these conditions, protein bodies are osmotically disrupted. Their content consisting mainly of storage and other proteins dissolves and can be separated from the membrane. Stirring was continued for 30 min, then the membrane suspension was subjected to sonication for further 30 min and centrifuged (30 min at 17 000 *g*). This treatment was repeated twice with the same buffer containing NaCl and twice without NaCl. Final purification was achieved with a buffer containing 5 M guanidinium chloride and with buffer alone.

Lipids were extracted from a membrane suspension with $CHCl_3$–MeOH (2:1). The extract was dried with Na_2SO_4, filtered, concd and subjected to TLC on silica gel F254 plates (Merck, Darmstadt). The plates were developed with $CHCl_3$–MeOH–H_2O (65:25:4). Lipids were detected by exposing the plate to I_2 vapour or by spraying it with 10% diphenylamine in EtOH–conc HCl–HOAc (1:5:4).

For the determination of phosphate, a sample was heated in a H_2SO_4–$HClO_4$ mixture until the organic matter was destroyed. The soln was then carefully neutralized with NaOH and P determined according to ref. [17].

Anti-pea lectin antibodies. Pea lectin was isolated from the albumin fraction (soluble at pH 5) of cotyledon extract by adsorption to Sephadex G-100 and elution with 0.25 M glucose similar to ref. [18]. After extensive dialysis, the preparation was electrophoretically pure and completely free from storage proteins. Polyclonal antibodies against pea lectin were raised in mice and purified by precipitation in half saturated $(NH_4)_2SO_4$ and passage over a column of DEAE-cellulose.

Marker protein. Pea lectin was determined by quantitative affinity chromatography on Sephadex G-100, α-mannosidase and other glycosidases by the method of Li [19] using *p*-nitrophenyl-glycosides as pseudosubstrates. NADH–cytochrome *c*–oxidoreductase was determined according to ref. [20] as modified in ref. [21].

Electrophoreses. Analytical SDS–PAGE of reduced and heat denatured samples were performed according to ref. [22] in polyacrylamide gels (1.25 or 10% T, 5% C) in a Mini-Protean II apparatus (Bio-Rad, München) in 1 mm gels and stained for protein with Coomassie Blue. M_r marker proteins were bovine serum albumin (66 000), hen ovalbumin (45 000), rabbit glyceraldehyde-3-phosphate dehydrogenase (36 000), bovine red cell carbonic anhydratase (29 000), bovine trypsinogen (24 000), soybean trypsin inhibitor (20 100), and bovine lactalbumin (14 200). For micropreparative electrophoresis, samples were subjected to SDS–PAGE as described. After this, gel regions containing the protein of interest were cut into 1 mm^3 dice with razor blades and subjected to electroelution in a Biotrap BT 1000 (Schleicher & Schuell, Dassel) according to ref. [23]. For Western blots, a semidry blotting apparatus (cti, Idstein) with graphite electrodes was used. Blotted nitro-cellulose membranes were dried at room temp. and stored at 4°. They were stained for lectin and lectin cross-reactive material with an immuno stain. For this, the membranes were washed in phosphate buffered saline (PBS) for 3 × 10 min, blocked by incubation in 1.5% fish gelatine for 1 hr, washed again and incubated overnight in the mouse anti-pea lectin antibody in PBS/0.5% Tween 20. After washing, the membranes were incubated for 1 hr with goat anti-mouse-IgG antibody-peroxidase conjugate in PBS/0.5% Tween 20, washed again and incubated for 30 min in peroxidase substrate soln consisting of 3-amino-9-ethylcarbazole and H_2O_2 according to ref. [24]. A positive reaction was indicated by a reddish-brown colour. Since the contrast of the bands was hardly visible on a photograph, the nitro-cellulose strips were documented by scanning in a Desaga CD50 TLC scanner. All blotting and staining experiments were accompanied by negative controls in which empty polyacrylamide gels after electrophoresis were blotted and the nitro-cellulose membranes treated as described. In these controls, no bands were observed (not shown). For staining of electrophoresis gels or blots for carbohydrate or glycoprotein the methods of refs [5, 6] were used.

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REFERENCES

1. van Driessche, E., Beeckmans, S., Dejaegere, R. and Kanarek, L. (1988) *Lectins, Biol. Biochem. Clin. Biochem.* **6**, 355.
2. McGee, C. M. and Murray, D. R. (1985) *J. Plant Physiol.* **120**, 1.
3. Mäder, M. and Chrispeels, M. J. (1984) *Planta* **160**, 330.
4. Lord, J. M., Kawaga, T., Moore, S. and Beevers, H. (1973) *J. Cell Biol.* **15**, 541.
5. Haselbeck, A. and Hösel, W. (1990) *Glycoconj. J.* **7**, 62.
6. Eckhardt, A. E., Hayes, C. A. and Goldstein, I. J. (1976) *Anal. Biochem.* **73**, 192.
7. Laurière, M., Laurière, C., Chrispeels, M. J., Johnson, K. D. and Sturm, A. (1989) *Plant Physiol.* **90**, 1182.
8. Weber, E., Süss, K. H., Neumann, D. and Manteuffel, R. (1979) *Biochem. Physiol. Pflanz.* **174**, 139.
9. Pusztai, A., Croy, R. R. D., Stewart, J. S. and Watt, W. B. (1979) *New Phytol.* **83**, 371.
10. Mettler, I. J. and Beevers, H. (1979) *Plant Physiol.* **64**, 506.
11. Kara, A. K. and Kindl, H. (1982) *Eur. J. Biochem.* **121**, 533.
12. Höfte, H., Faye, L. Dickinson, C., Herman, E. M. and Chrispeels, M. J. (1991) *Planta* **184**, 431.
13. Kaminski, P. A., Buffard, D. and Strosberg, A. D. (1987) *Plant Molec. Biol.* **9**, 497.
14. Schmidt, M. F. G. (1989) *Biochim. Biophys. Acta* **988**, 411.

15. Sinensky, M. and Lutz, R. J. (1992) *BioEssays* **14**, 25.
16. Ferguson, M. A. J. and Williams, A. (1988) *Annu. Rev. Biochem.* **57**, 285.
17. Martin, J. B. and Doty, D. M. (1949) *Anal. Chem.* **21**, 965.
18. Trowbridge, I. S. (1974) *J. Biol. Chem.* **249**, 6004.
19. Li, Y. T. (1967) *J. Biol. Chem.* **242**, 5474.
20. Martin, E. M. and Morton, R. K. (1956) *Biochem. J.* **62**, 69.
21. Schnarrenberger, C., Oeser, A. and Tolbert, N. E. (1972) *Planta* **104**, 185.
22. Laemmli, U. K. (1970) *Nature* **227**, 680.
23. Jacobs, E. and Clad, A. (1986) *Analyt. Biochem.* **154**, 583.
24. Endl, J., Xu, H. and Peters, J. H. (1990) in *Monoklonale Antikörper* (Peters, J. H. and Baumgarten, G., eds), p. 363, Springer, Berlin.