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POLYGALACTURONASE INHIBITORS IN BEAN PODS

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Key Word Index—*Phaseolus vulgaris* L.; Fabaceae; bean; polygalacturonase-inhibiting protein; pathogen resistance.

Abstract—The amount of polygalacturonase-inhibiting protein (PGIP) was 14 times higher in bean pods than in etiolated hypocotyls. The PGIP was extracted from bean pods and partially purified by chromatography on columns of S-Sepharose, DEAE-Sephadex A-50, and Sephadex G-75. Further purification by ion-exchange chromatography on a Mono Q column separated two isoforms of the inhibitor. The two PGIPs were similar in most properties but differed slightly in pI values. They also differed in one residue of the *N*-terminal amino acid sequences. Both bean pod PGIPs differed in two and possibly three residues of the deduced *N*-terminal amino acid sequence for hypocotyl PGIP. Small alterations in the structure of PGIP may represent a strategy in bean plants for resistance to a variety of pathogens.

INTRODUCTION

The first evidence for a polygalacturonase-inhibiting protein (PGIP) in plants was obtained in 1953 by Weurman [1] who found a thermolabile, and presumably proteinaceous, inhibitor of fungal pectinase in the sap of pears. Abu-Goukh *et al.* [2] confirmed the presence of PGIP in pears and showed that the activity was due to several isoforms of PGIP that inhibit PGs from various fungi including *Botrytis cinerea*. PGIPs have been detected in vegetative tissues of numerous dicots [3–6] but it is becoming evident that these proteins are also common in fruit tissues. They have been found in apples [7], cucumbers [8], peppers [9], raspberries [10], tomatoes [11] and pears. I have now discovered PGIP in the pericarp of bean fruits, and the level of PGIP in the pods is much higher than in bean hypocotyls and other vegetative tissues. This paper describes the isolation of PGIP from bean pods, separation of two isoforms and some of their properties.

RESULTS AND DISCUSSION

Relative amounts of PGIP in bean hypocotyls and pods

Reported values for PGIP levels in bean hypocotyls were obtained by assays with PG from *Colletotrichum lindemunthianum* [3] and with PG from *Aspergillus niger* using a viscometric method [12]. To make a direct comparison of the levels of PGIP in hypocotyls and pods, an extract of bean hypocotyls was prepared the same way as for pods. By assaying PGIP in the crude extract, bean hypocotyls were found to contain 14.8 units PGIP g^{-1} fresh wt. This low value is consistent with the report by Toubart *et al.* [6] that only 30 μg of PGIP was purified from a kg of hypocotyls. The amount of PGIP in bean pods was 210 units g^{-1} (Table 1) or about 14 times greater than that in hypocotyls.

Purification of PGIP

The purification of bean pod PGIP is summarized in Table 1. The most effective step in the procedure was ion-exchange chromatography on S-Sepharose (Fig. 1). About 80% of the proteins in the crude extract eluted in the flow-through and had no PGIP activity. PGIP was then eluted as a small peak of protein just before a much larger peak of protein that was devoid of activity (Fig. 1). Further purification of the inhibitor was achieved by chromatography on DEAE-Sephadex A-50 and Sephadex G-75 (elution profiles not shown). An additional impurity (fractions 20 and 21) was removed by chromatography on the Mono Q column (Fig. 2). The remaining protein that eluted off the Mono Q column contained PGIP activity and consisted of a large peak (designated PGIP I) followed by a smaller peak (PGIP II) and tailing-off of activity with a possible small peak at fraction 54.

Properties of PGIPs

The M_rs of PGIP I and of PGIP II were determined by gel permeation on Sephadex G-75 and both were found to be *ca* 44 k, which is the same value reported for bean hypocotyl PGIP [12]. SDS–PAGE of the purified PGIPs revealed that they consisted of single polypeptides but with apparent M_rs each of only about

Table 1. Purification of bean pod PGIP

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units mg^{-1})
Crude extract	2370	210 000	89
S-Sepharose	17.8	200 000	11 200
DEAE-Sephadex A-50	12.4	165 000	13 300
Sephadex G-75	10.7	152 000	14 200
Mono Q			
PGIP I	4.1	97 600	23 800
PGIP II	1.4	31 400	22 400

38 k. A similar lower value for hypocotyl PGIP was obtained by SDS–PAGE than by gel permeation [12]. The predicted M_r of the mature PGIP from the DNA sequence of the recently cloned PGIP gene is 33 984 [6]. The *N*-terminal amino acid sequences of PGIP I and PGIP II are presented in Fig. 3 along with the sequence deduced from the cloned PGIP gene [6]. In the first 28 amino acids, PGIP I and PGIP II differed at amino acid 27 and possibly the first amino acid. Both bean pod PGIPs differed from the deduced sequence [6] at not only amino acid 27 but also 25. In addition, the results indicate that PGIP I may begin with an aspartic acid residue rather than glutamic acid [6] but this amino acid was not identified for PGIP II.

PGIP I and PGIP II were equally effective inhibitors of *Aspergillus niger* PG, with essentially the same specific activities for the purified inhibitors when assayed with this enzyme (Table 1) They also inhibited the PG from *A. japonicus* (Pectolyase, Sigma Chemical Co.) but not the PGs from *Rhizopus* sp. (Macerase, Calbiochem) or tomato fruit. The inhibitors had similar heat stability, with 50% loss of activity by heating at 63° for 5 min, and they were most effective at pH 4.7. The pIs determined by chromatofocusing on a Mono P column (Pharmacia) were 9.1 and 9.2 for PGIP I and PGIP II, respectively.

Isoforms of PGIP are known to exist in other plant tissues. Con A chromatography of protein extracts from tomato pericarp yielded a major fraction of PGIP that bound to Con A and a minor fraction that did not bind to the lectin [11]. Short segments of the two proteins were found to have similar amino acid sequences. The similarities in the peptides but differences in M_r and Con A binding indicated that tomato PGIPs may differ only in glycosylation [11]. The isoforms of PGIP in pears are similar in M_r but differ in ionic properties

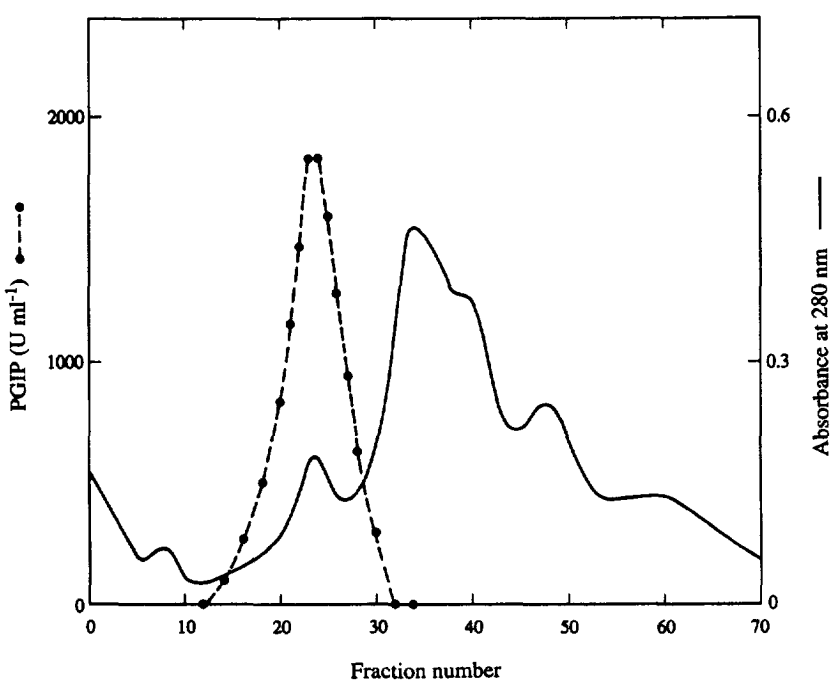

Fig. 1. Chromatography of a crude extract of bean pods on a 2.5 × 15 cm column of S-Sepharose Fast Flow equilibrated with 0.05M MES, pH 6.0. Elution was conducted with 1000 ml of a linear gradient of 0–0.4 M NaCl in 0.05 M MES, pH 6.0. Fraction size was 10 ml.

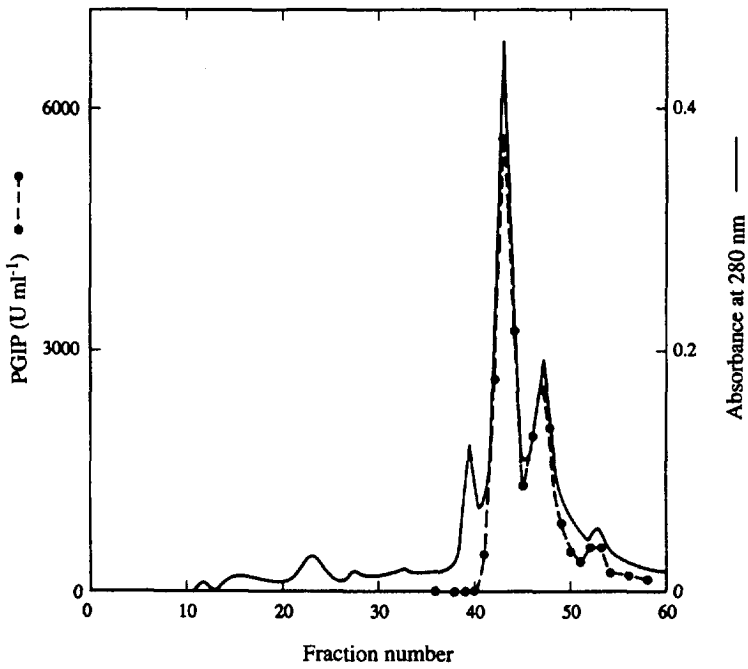

Fig. 2. Chromatography of partially purified PGIP on a mono Q HR 5/5 column equilibrated with 0.02 M CAPS, pH 10. Fraction size was 1 ml.

with pIs of 4, 5, 6.6, and 7.7 [2]. The differences in pIs suggest differences in amino acid compositions of the isoforms although only the major pear PGIP has been sequenced [13].

PGIPs have been implicated in resistance to fungal pathogens in plants [3] and there is some evidence to support this. A decrease in PGIP in pears was correlated with a decline in fruit resistance to several organisms [14]. A similar decrease in PGIP in ripening raspberries was inversely correlated with the ability of *Botrytis cinerea* to colonize the fruit [10]. Powell *et al.* [15] recently expressed pear PGIP in transgenic tomatoes and found that resistance to *B. cinerea* in fruit of primary tomato transformants correlated with the amount of PGIP expressed in the fruit. However, PGIPs were not the determinant of monogenic resistance in plant–pathogen relationships examined [4, 16, 17] rather, they were regarded as a general host resistance phenomenon. There is further inconsistency in a role for PGIPs in disease resistance in the inhibition of PG from *A. niger* which is not a plant pathogen. The association of PGIPs with fruit tissues suggests possible roles in fruit development but there is currently no evidence to support this view.

Bean Pod PGIP I	DLXNPQDKQALLQIKKDLGNPTTLLSSLP
Bean Pod PGIP II	XLXNPQDKQALLQIKKDLGNPTTLLSQLX
Bean Hypocotyl PGIP	ELCNPQDKQALLQIKKDLGNPTTLSSWLP

Fig. 3. Comparison of the *N*-terminal amino acid sequences of PGIP I and PGIP II with the sequence of bean hypocotyl PGIP published by Toubart *et al.* [6].

EXPERIMENTAL

PGIP purification. Plants of bean (*P. vulgaris* L.) cv. Blue Lake were grown outdoors. One kg of bean pods were harvested when the seeds were small and immature and homogenized in 1.5 l of 1 M NaCl using VirTis and Polytron homogenizers. The slurry was adjusted to pH 7, stirred for 30 min and centrifuged. The supernatant was adjusted to pH 3 by addition of 1 M HCl and the ppt. that formed was removed by centrifugation and discarded. The soln was adjusted to pH 6 and solid $(NH_4)_2SO_4$ was added to 75% of satn. The ppt. was collected by centrifugation, dissolved in H_2O and dialysed against 0.05 M NaOAc, pH 6, for 16 h.

The dialysed extract was applied to a 2.5 × 15 cm column of S-Sepharose Fast Flow (Pharmacia) equilibrated with 0.05 M MES, pH 6 and then eluted with 1 l of a linear gradient of 0–0.4 M NaCl in 0.05 M MES, pH 6. The frs collected were assayed for PGIP and those containing the inhibitor were pooled, concd to 10 ml by ultrafiltration using a YM 10 membrane (Amicon Corp.) and dialysed against 0.15 M NaCl. The soln was applied to a 2.5 × 94 cm column of DEAE-Sephadex A-50 equilibrated with 0.02 M MES, pH 6, containing 0.13 M NaCl and eluted the equilibration buffer. The frs containing PGIP were pooled, concd to 8 ml by ultrafiltration and applied to a 2.5 × 95 cm column of Sephadex G-75 equilibrated with 0.15 M NaCl. The frs containing the highest PGIP were concd to 5 ml and dialysed against 0.02 M NaCl. One ml aliquots of the soln were diluted with 1 ml of 0.02 M CAPS, pH 10, and loaded onto a Mono Q HR 5/5 column (Pharmacia). The column was eluted with 30 ml of a linear gradient of 0–0.2 M NaCl in 0.02 M

CAPS, pH 10 at 1 ml min^{-1}. The frs containing PGIP were pooled, adjusted to pH 6 and dialysed against 0.15 M NaCl.

PGIP in bean hypocotyls. Blue lake beans were sown in trays of vermiculite, watered and allowed to germinate in the dark. The hypocotyls were harvested after 8 days (*ca* 5 cm tall) and extracted by homogenizing in 1 M NaCl. The proteins in the extract were pptd with $(NH_4)_2SO_4$, dissolved in H_2O and dialysed against 0.05 M NaOAc, pH 6, as described above for bean pods.

PG purification. Twenty ml of pectinase (Sigma) was diluted with 40 ml of 0.05 M NaOAc, pH 5.5, and ultrafiltered to 20 ml using a PM10 membrane (Amicon Corp.). The dilution and ultrafiltration was repeated $\times 4$ times to remove glycerol and salts from the enzymes. The soln was then applied to a 2.5×20 cm column of Q-Sepharose Fast Flow (Pharmacia) equilibrated with 0.05 M NaOAc, pH 5.5. Elution was with 1 l of a linear gradient of 0–0.4 M NaCl in 0.05 M NaOAc, pH 5.5. The largest peak of PG eluted at 0.09 M NaCl and this enzyme was used for assaying PGIP. A unit of PG was defined as that amount that catalysed the formation of 1 μmol reducing groups per 30 min at 37° from polygalacturonic acid at pH 4.5.

PGIP assay. PGIP was assayed by measuring its effect of reducing the activity of *A. niger* endo-PG. The reaction mixture consisted of 0.1 ml of PG (1 unit), 0.2 ml of 0.2 M NaOAc, pH 4.5, 0.2 ml of diluted PGIP soln and 0.5 ml of 1% polygalacturonic acid, pH 4.5. After 30 min at 37°, the solns were heated at 100° for 5 min to stop the reactions, cooled, and analysed for reducing groups [18]. A unit of PGIP is defined as that amount required to reduce to 50% the activity of one unit of PG.

Other methods. Protein concns were measured according to ref [19]. SDS–PAGE was conducted on PhastGel gradient 10–15 gels in a PhastSystem (Pharmacia) and stained with Coomassie blue. *N*-terminal protein sequences were determined on a model 470A protein sequencer (Applied Biosystems) by Dr John Wunderlich, University of Georgia.

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