



Identification of soybean elicitation competency factor, CF-1, as the soybean Kunitz trypsin inhibitor

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The soybean defence responses that occur in cells immediately adjacent (proximal) to the hypersensitive response (HR) can be induced by the cell wall glucan elicitor (WGE) from *Phytophthora sojae*, but in some cultivars only in cells that have been pre-conditioned by endogenous competency factors associated with wounded or HR-dying cells. Competency factor, CF-1, causes a general amplification of phenylpropanoid responses to elicitor and greatly enhances the phenolic polymer response to elicitor. CF-1 activity, which is heat-labile and associated with a low speed centrifugal pellet of soybean tissues, can be partially mimicked by reduced glutathione (GSH). Here it is reported that GSH releases CF-1 activity into intercellular washing fluids, suggesting it is associated with the apoplast. Likewise, GSH can be used to enrich CF-1 activity greatly in extracts from low speed pellets. This enrichment allowed the purification of CF-1 to near homogeneity and its subjection to mass spectral and sequence analysis. CF-1 was identified as the soybean Kunitz trypsin inhibitor (SKTI). Although plant protease inhibitors have been implicated in insect and pathogen resistance, to the authors' knowledge this is the first suggestion of a regulatory role in activation of cellular defence responses. Protease inhibitors play a role in many signaling processes in eukaryotic systems, and SKTI has intriguing three-dimensional homologies to interleukin 1- α and 1- β and fibroblast growth factor.

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INTRODUCTION

The accumulation of antimicrobial pterocarpan phytoalexins, the glyceollins, is a major defence mechanism hypothesized to provide resistance to soybean [*Glycine max* (L.) Merrill] tissues against infection by *Phytophthora sojae* [Kauf. and Gerde.] [5, 6]. Both the timing and magnitude of accumulation of glyceollin in soybean tissues infected with *P. sojae* correlate well with differential resistance of various soybean cultivars to races of this pathogen [6, 14, 15, 33]. Race-cultivar specific resistance in soybean tissues is conditioned by several dominant host *Rps* genes (14 described at seven different loci) against several known races of the pathogen [29]. However, only a handful of these interactions have been rigorously investigated for the correlation of glyceollin accumulation to resistance.

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Abbreviations used in text: GSH, glutathione; HR, hypersensitive response; IWF, intercellular washing fluids; LSP, low speed centrifugal pellet; SKTI, soybean Kunitz trypsin inhibitor; WGE, wall glucan elicitor.

In addition, pre-formed conjugates of the isoflavones, genistein and daidzein, are hydrolysed at the infection front in *P. sojae* infected cotyledons [14]. This hydrolysis leads to the free aglycones of daidzein, the first committed precursor of glyceollin [5], and of genistein, which has been shown to possess antibiotic activity against *P. sojae* [27]. Moreover, phenolic polymer deposition occurs in tissues of resistant lesions [24], and several other potential phytoalexins other than the pterocarpan glyceollin have been characterized [18]. Predominant among the other potential phytoalexins are other pterocarpan and the coumestan, coumestrol. Interestingly, all of these alternative phytoalexins are also derived from daidzein. Taken together, these studies suggest that there are potentially multiple defence responses induced in an incompatible lesion, including the formation of glyceollin and other phytoalexins, the release of a possible pre-formed antibiotic (genistein) and the formation of possible cell wall associated barriers to pathogen spread.

The multiplicity and coordination of these potential defence responses have been more thoroughly elucidated at the host level through the investigation of cellular responses to cell-free elicitors from the pathogen. In particular, glyceollin and coumestrol accumulation,

phenolic polymer deposition and changes in the net levels of isoflavone aglycones and their conjugates can all be induced by the cell wall glucan elicitor (WGE) from *P. sojae* [8]. However, glucan elicitor preparations optimally trigger glyceollin accumulation and phenolic polymer deposition in some soybean cultivars only in cells that have first been pre-conditioned by competency factors associated with hypersensitive cell death or wounding [10].

Two wound-released signals of host origin (CF-1 and CF-2) have been described that are involved in the establishment of elicitation competency for the glucan elicitor [10]. CF-1 enhances all phenylpropanoid responses proximal to the point of elicitor treatment, particularly the phenolic polymer response, whereas CF-2 is specifically required for the glyceollin response to elicitor. The effects of CF-1 and CF-2 can be mimicked by reduced glutathione (GSH) and orthovanadate, respectively [10, 12]. Elicitation competency for these proximal cellular responses can also be established by wound exudates [10, 12], which has allowed some initial fractionation of the active factors. CF-1 is heat labile and associated with low speed centrifugal pellets from wounded soybean tissues, whereas CF-2 is low molecular weight and heat stable, but chemically very unstable, which has made its further fractionation difficult [10].

A minimal wound, snapped cotyledon assay has further allowed the clear separation and characterization of individual signaling responses to wounding, light and glucan elicitor treatment, conditions and signals which are often superimposed upon one another in other assays [12]. This work demonstrated that light and glucan are the primary signals for genistein and daidzein accumulation, respectively. Wounding, which releases CF-1 activity, appears to be the primary signal initiating phenolic polymer deposition, a process greatly amplified in the presence of the glucan elicitor [12]. As noted below, the initiation of phenolic polymer deposition can thus be used as a very sensitive assay for CF-1.

In this paper the finding that the CF-1 mimic, reduced GSH, can dramatically enrich soluble cell free extracts for endogenous CF-1 activity was described. This greatly facilitated the subsequent fractionation, purification and identification of CF-1 as the soybean Kunitz trypsin inhibitor (SKTI). To the authors' knowledge, this is the first example of a protease inhibitor being proposed to play a regulatory role in the deployment of host-pathogen defence reactions in plants.

MATERIALS AND METHODS

Chemicals

The intact WGE was prepared from the cell walls of race 1 of *P. sojae* (Kauf. and Gerde.) according to Ayers *et al.* [1]

and as described previously [11]. Before use, the unfractionated and insoluble wall glucan preparation was sonicated and then autoclaved for 3 h in deionized double distilled water [1].

Reduced GSH and metavanadate were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). A stock solution of metavanadate was prepared at 50 mM in sterile DDW and kept at room temperature. At this concentration, orthovanadate is formed within 24 h and is stable indefinitely. Orthovanadate was diluted to the appropriate concentrations immediately before the experiment. The final concentration of orthovanadate used was 90 μM .

Growth of soybean seedlings

Soybean seeds [*Glycine max* L. (Merr.) cvs. Williams and Harosoy] were kindly provided by Dr A. F. Schmitthenner (OARDC, Wooster, OH, U.S.A.). Seedlings were grown as described previously [14] with slight modifications. Instead of vermiculite, seedlings were grown in Metromix 360 (Sierra Grace, Milpitas, GA, U.S.A.) at 26°C with 500 $\mu\text{E m}^{-2} \text{s}^{-1}$ of light and a 14 h photoperiod. The flats were immediately watered very thoroughly for germination. After 3 days, the plants were watered every other day from the top. Plants were not fertilized. Unless otherwise noted, cotyledons from 8 day old plants were used.

Cotyledon assays

Cotyledons from 8 day old seedlings, unless otherwise noted, were harvested in small batches and used immediately. The cut cotyledon assay [11] and minimal wound snapped cotyledon assay [12] were performed as described previously.

Collection of intercellular washing fluids from cotyledon tissues

Cotyledons were cut into four equal sections to facilitate vacuum infiltration. The cut sections were washed in two changes of distilled water prior to infiltration to remove wound exudate from the cut surfaces and were then wrapped in cheesecloth and placed in a beaker with the infiltration treatment. A second, smaller beaker was used to keep the tissues submerged under the treatment. The assembly was then placed in a vacuum dessicator and vacuum pulled by aspirator until bubbling from the tissues ceased (approx. 5 min). The vacuum was then slowly released. Successful infiltration was seen as a complete water soaking of the tissues. The cotyledon sections were then put into nested centrifuge tubes, in which the inner tube (holding the tissue) had holes in the bottom to allow drainage of the intercellular washing fluids (IWF) into the

outer tube. Centrifugation was carried out at very low speeds (100 *g* for 5 min) to collect the IWF.

Alternatively, IWF was collected by needle infiltration. After cutting off 1 mm of the petiole end of individual cotyledons, a syringe with a 28 gauge needle was inserted in the opposite end of the cotyledon and used to infuse the tissue with the treatment. As infiltration proceeded, the IWF dripped out of the lower, previously cut end. The first two drops were discarded and the next 100–200 μ l were collected as IWF.

Fractionation of soybean extracts and extraction of CF-1 from low speed centrifugal pellets

For initial studies, soybean cotyledon tissues were ground in microfuge tubes using a polypropylene pestle with 2 ml g^{-1} tissue of extraction buffer, 25 mM citrate–50 mM phosphate buffer (pH 7.0). All operations were carried out at 4°C. The extract was centrifuged at 10 000 *g* for 10 min. The low speed pellet was collected and the supernatant further centrifuged at 75 000 *g* for 30 min. Consistent with the IWF results, approx. 90 % of CF-1 activity was found in the low speed pellet which contains cell wall fractions of the cell. Its activity when applied as a suspension in the cut cotyledon assay suggested that the active CF-1 component(s) were released during the assay. Indeed, small amounts of CF-1 activity could be solubilized simply by allowing the low speed pellet to sit in excess 25 mM citrate–50 mM phosphate buffer (pH 7.0) at 4°C for 20 min. To facilitate purification, however, a series of different methods for the solubilization of CF-1 from the low speed pellet were compared. Included were 500 mM sodium chloride, 100 mM calcium chloride, 10 mM EGTA, 4 mM DTT and 1 mM GSH. Most methods did not effectively release CF-1 activity. Sodium chloride led to slightly more solubilization than the buffer alone, but reduced GSH led to the highest enrichment, generally giving at least a five-fold increase in soluble CF-1 activity over buffer. The optimal conditions for GSH extraction were 5 mM GSH for 20 min. In the final extraction protocol, 100 gm cotyledon tissues were ground using a Sorvall Omnimixer in 200 ml 25 mM citrate–50 mM phosphate buffer (pH 7.0). The extract was filtered through cheesecloth to remove unbroken cells and the extract then centrifuged at 10 000 *g* for 10 min. This pellet, the low speed centrifugal pellet (LSP), was incubated at 4°C with 5 mM GSH for 20 min. The suspension was centrifuged at 14 200 *g* for 5 min and the supernatant used for the further purification of CF-1.

Column chromatography

Chromatography was first performed on a BioRad Econo-Pac Mini High-Q anion exchange cartridge (bed

volume 5 ml). Fifteen ml GSH extracted LSP was dialysed against 25 mM citrate–50 mM phosphate buffer (pH 7.0) prior to application to the Mini High-Q column. After sample application and washing into the column with buffer, a linear gradient (200 ml) of 0–1 M sodium chloride in the same buffer was applied to the column. To minimize the use of individual fractions, protein concentration was followed at 280 nm. Scale-up was accomplished with a 25 ml High-Q column. For this column, 50 ml of the extract was loaded on the column and the gradient elution was accomplished from 0 to 1 M sodium chloride in 800 ml. Further fractionation was accomplished by gel exclusion chromatography in extraction buffer on a Sephadex G-50 column with a bed volume of 40 ml.

Electrophoresis, mass spectral analysis and sequencing

Native and SDS gel electrophoresis were performed according to Laemmli [22]. Both were performed using a Mini-V 8.10 vertical gel apparatus (Life Technologies Inc., Gaithersburg, MD, U.S.A.). To maximize sensitivity and thus minimize use of sample, gels were usually stained with the silver stain. For sequencing, protein bands were detected using Coomassie blue R-250 using a protocol with reduced methanol to minimize fixation of the protein [M. A. Gawinowicz, pers. comm.]. The gel was not fixed, but stained directly for 30 min in 20 % methanol and 0.5 % acetic acid containing 0.05 % Coomassie blue. The gel was destained in 30 % methanol until the protein band was visible. The band was then cut out and immediately sent for mass spectral analysis and sequencing. Protein mass spectral analysis and internal sequencing were performed by Mary Ann Gawinowicz at the Protein Chemistry Core Facility of the Howard Hughes Medical Institute of Columbia University (New York, NY, U.S.A.). The protein was reduced, alkylated with *N*-isopropyliodoacetamide and digested with endoproteinase Lys-C. MALDI-MS was done on 7.5 % of the total digest. Subsequently HPLC was performed on the remaining digest and a clearly resolved peak of mass *M* (+*H*) 1492.74 was sequenced.

For CF-1 activity measurements, zones or bands were excised from 5 % native gels using a razor blade to cut out the area of interest, followed by electroelution into dialysis bags (Spectra/Por, 2000 Da cutoff, Baxter Scientific) attached to the tray of a flat bed electrophoretic cell (Model H4, Life Technologies, Gaithersburg, MD, U.S.A.). Native running buffer was used to submerge the dialysis tubing and electroelution was performed for 2 h at 100 V. After removal of the gel piece, the electroeluted samples were first dialysed against extraction buffer and then against water. This sample was then lyophilized and used for SDS electrophoresis or for assay of CF-1 activity.

Measurement of CF-1 in phenolic polymer analyses

CF-1 activity was followed by its ability to initiate phenolic polymer deposition in the cut cotyledon assay. After exploring several potential assays, with and without glucan, this proved to be the most convenient and sensitive assay for use in following the multiple fractions generated. Samples were always dialysed against water and lyophilized just before analysis because of the interference of GSH and buffer with the assay. The assay was always performed on the sample in serial two-fold dilutions starting with a 1:2 (v/v) dilution based on the original sample volume. This approach takes both the lowest level of detection and saturation of the bioassay into account, and thus allowed the best comparisons of the relative concentrations of CF-1 activity in various fractions and after various treatments. Phenolic polymers were measured by u.v. analysis as described below. In all cases, ferulic acid was used as a standard and CF-1 activity is thus expressed as ferulic acid equivalents.

The u.v. assay for phenolic polymers is a rapid assay that gives results very parallel to the more difficult thioglycolic acid procedure {[9], T. L. Graham, unpublished work}. It was carried out by extracting the pooled proximal cell layers from 10 treated soybean cotyledons in 80 % ethanol (50 mg/400 μ l) as described elsewhere [7]. The pellet was drained and resuspended in 1 ml of 95 % ethanol and allowed to soak overnight. A second 95 % ethanol wash (1 ml) was then carried out for at least 30 min and the pellet collected by centrifugation at 13 000 g for 5 min. The pellet was finely suspended in 1 ml of 1.25 M sodium hydroxide. This mixture was vortexed three to four times over the next few hours and then left for a standardized time of 20 h for extraction at room temperature. The sodium hydroxide saponifies esterified phenolics and also extracts high molecular weight phenolic polymers. Samples were vortexed and centrifuged at 13 000 g for 5 min. The absorbance (320 nm) of each extract was taken by adding 100 μ l of extract to 900 μ l of 1.25 M sodium hydroxide. A standard curve was made using ferulic acid and values were expressed as mg ferulic acid equivalents per gram fresh weight tissue.

Unless otherwise noted, CF-1 activity was standardized for comparison as the mg ferulic acid equivalents per gram tissue in the cut cotyledon assay using serial dilution of the CF-1 sample being analysed. Since each CF-1 assay commonly used at least 1/4th of the volume of a given column fraction, sample was highly limited. Thus the protein was estimated by absorbance at 280 to minimize the use of sample, using bovine serum albumin as a standard ($A_{280} = 0.64$ for a 1 mg ml⁻¹ solution). For individual fractions, specific activity was thus expressed as CF-1 activity per mg protein. To further save samples, fractions were sometimes pooled into peaks for analysis. In this case, specific activity was more arbitrarily expressed as

CF-1 activity divided by the area under the peak in integration units.

RESULTS

Presence of CF-1 in IWF

Preliminary characterization of CF-1 suggested that it was proteinaceous and possibly associated with cell wall fractions [10]. Moreover, it was known that GSH mimicked CF-1 activity, which could mean that GSH either activated or released endogenous CF-1. Simple vacuum infiltration of soybean cotyledons with 1 mM GSH greatly increased CF-1 activity in IWF (over 10-fold) when compared to infiltration with water alone. An SDS gel of water and GSH infiltrates demonstrated that GSH selectively solubilized a discrete array of protein bands, with particular enrichment of a major band at about 21 kDa. GSH extraction of the low speed pellet from soybean tissue extracts showed a similar protein banding pattern, and it was thus decided to employ this extract for further purification.

Preliminary characterization of CF-1 by electroelution from native gels

To gain some initial idea of the relative size of CF-1, 200 μ l of the standard GSH extract was applied to 5 % native gels using a preparative comb. At first, the gel was cut into three major sections and electroeluted to test for activity. The lower section included the lower 1/5th of the gel, while the upper and middle sections were equal sections from the remainder of the gel. In a subsequent experiment, the middle section was further cut into three subsections and the lower section into two subsections. The CF-1 activities and a corresponding SDS gel of the electroeluted samples are shown in Fig. 1. As can be seen, nearly all of the CF-1 activity was associated with the lower section of the gel, which contained the major protein band at approx. 21 kDa.

Fractionation of CF-1 activity upon anion exchange chromatography

Owing to its high capacity, ion exchange is often the method of choice for initial protein fractionations. In view of the small amounts of protein available the authors began with Mini High-Q columns. To further maximize yield from these small columns, they were purposely overloaded. Fractionation of CF-1 activity on Mini High-Q anion exchange led to several major peaks. Fig. 2 shows a typical run. At first, part of the fractions from these peaks were pooled into peaks "O" (non-binding) and "A-E" for analysis. The majority of total CF-1 activity was found to be associated with peaks O and B

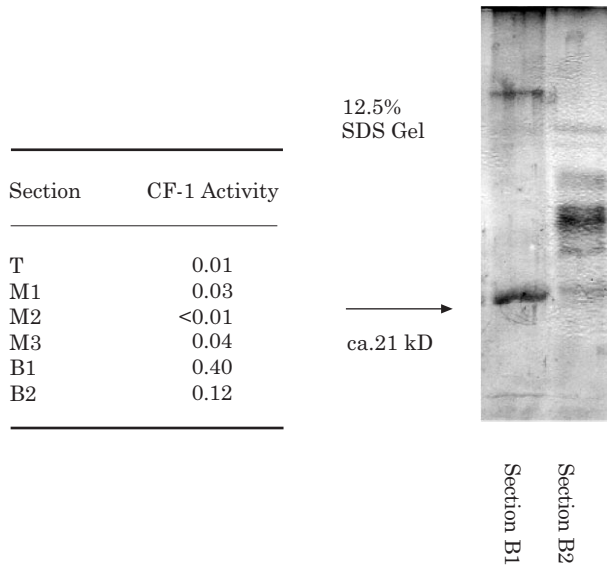


FIG. 1. Activities and SDS electrophoretic gel (12.5% acrylamide) on proteins eluted from sections of a 5% native acrylamide gel on the GSH extract of the low speed pellet. The lower 1/5th of the gel was cut for section B (bottom), while equal sections of the remainder of the gel made up sections M (middle) and T (top). Sections B and M were cut into two and three additional equal sections as noted. Gel pieces were electroeluted, tested for CF-1 activity (table) and run on a 12.5% SDS gel (data only shown for B1 and B2).

(Table 1). Electrophoresis of fraction O showed it to contain nearly all protein bands in the original extract, consistent with overloading of the column. The relative specific activity of peak B was 4.4 times that of peak O. Individual fractions within peak B were then analyzed for CF-1 activity. Fraction 37, eluting at approx. 0.14 M NaCl, was found to have the highest specific activity (Table 1). The relative specific activities of fractions 37, 40 and 43 appear to correlate very well with the major protein band at *c.* 21 kDa (inlay, Fig. 2).

Scale-up purification of CF-1

To purify larger quantities of CF-1 for possible mass spectral and sequence analysis, a larger High-Q column was used. Altogether, three preparative runs were made that all gave similar fractionations and results. A representative run is described here. Protein binding to this larger column was essentially complete (Fig. 3). Resolution on the larger column was less good, but nonetheless activity was associated with a major peak, HQ-A, again eluting at about 0.15 M NaCl (Fig. 3, Table 2). This time there was much less non-binding protein and peak HQ-A had 22 times the specific activity of peak HQ-O. Peak HQ-A was pooled and further chromatographed on a Sephadex G-50 column. An excellent separation was

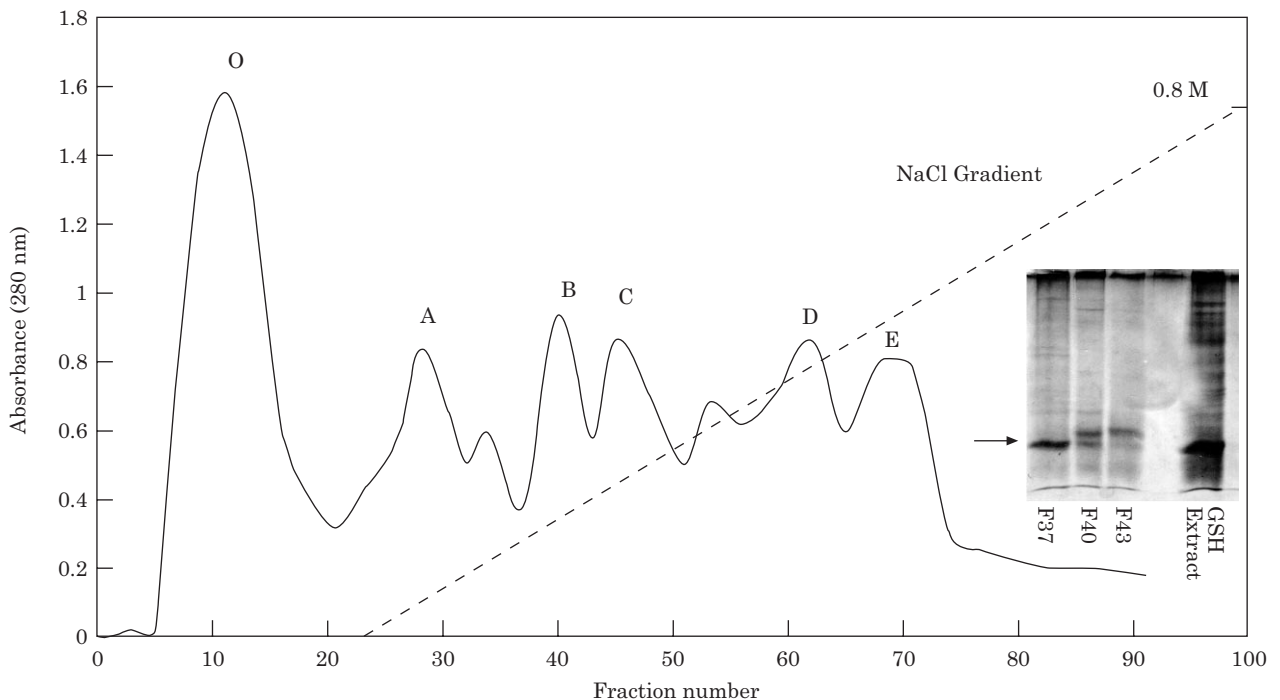


FIG. 2. Elution profile and SDS gel on fractions from a Mini High-Q anion exchange column on the GSH extract from the low speed pellet. The highest specific activity for CF-1 was found in peak B. The SDS electrophoresis gel (10% acrylamide, inlay) shows the protein banding pattern for the original GSH extract and for fractions 37, 40 and 43, which were at the beginning, middle and end of peak B. See Table 1 for CF-1 activity data.

TABLE 1. CF-1 activities in fractions from Mini High-Q column in Fig. 2

Fraction	CF-1 activity*	Specific CF-1 Activity†
O	1.7	27
A	0.8	29
B	2.2	118
C	0.7	34
D	0.5	16
E	0.1	4.0
37	1.1	42.2
40	0.7	17.2
43	0.2	2.8

*mg ferulic acid equivalents per gm tissue as measured in a 1:2 dilution.

†For fractions, this is CF-1 activity per mg protein; for peaks, this is CF-1 activity per area under the peak in integration units.

obtained (Fig. 4) and the highest specific activity (Table 2) was associated with a minor protein peak (peak III). The specific activity of peak III was 5.8 times the specific activity of HQ-A. This peak again showed a single major band of about 21 kDa (inlay, Fig. 4). Based on protein concentrations estimated by absorbance at 280 nm, the final specific activity of peak III was 39.7 per mg protein as compared to 1.6 per mg protein in the GSH extract from the low speed pellet. This represents approx. a 25-fold purification. Although this may seem low, it must be remembered that the CF-1 protein is already very highly enriched in the “crude” extract, first by isolation of the low speed pellet and secondly by highly selective GSH solubilization from the pellet.

Mass spectral analysis of CF-1

Peak III from Fig. 4 was run on a final SDS gel and stained with the sequencing Coomassie stain. The band at

TABLE 2. CF-1 activities in fractions from preparative High-Q and G50 columns

Fraction	CF-1 activity	Specific CF-1 activity
HQ-O	0.01	2
HQ-A	0.83	44
HQ-B	0.42	12
HQ-C	0.02	<1
G50-I	0.33	12
G50-II	0.31	116
G50-III	0.31	256

21 kDa was cut out and subject to mass spectral analysis and internal sequencing as described in Materials and Methods. The first attempt at mass spectral analysis yielded no peaks on HPLC after digestion with trypsin. Subsequent attempts at digestion of crude CF-1 preparations with trypsin (monitored by SDS gel electrophoresis) showed that the band at 21 kDa was highly resistant to digestion by trypsin. It was thus attempted to digest and obtain MS data on peak III using endoproteinase Lys-C. Digestion yielded excellent results with MALDI mass spectroscopy. The masses of five fragments in the range of approx. 1000–3000 Da were obtained as shown in Table 3. After correcting for the increased masses of cysteine residues caused by alkylation by *N*-isopropyl iodacetamide, all five fragments matched those predicted from the primary sequence of variant A of the SKTI (Table 3).

The identity of CF-1 as a variant of the SKTI was further confirmed by amino acid sequencing of the purified fragment of mass 1492.45. A second mass spectral on the purified fragment yielded 1492.74, even closer to the predicted value of 1492.75. The amino acid sequence of the fragment confirmed the mass spec data and yielded a sequence with 100 % identity to that predicted (Table 3).

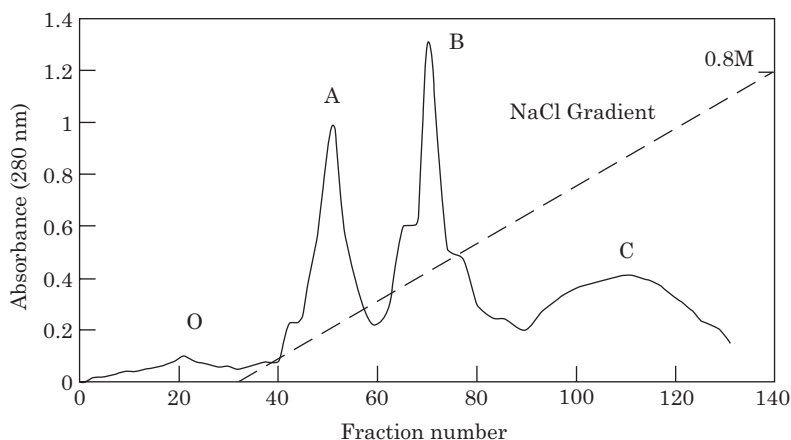


FIG. 3. Elution profile of fractions from a preparative High-Q anion exchange column on the GSH extract from the low speed pellet. The highest specific activity for CF-1 was found in peak A. See Table 2 for CF-1 activity data.

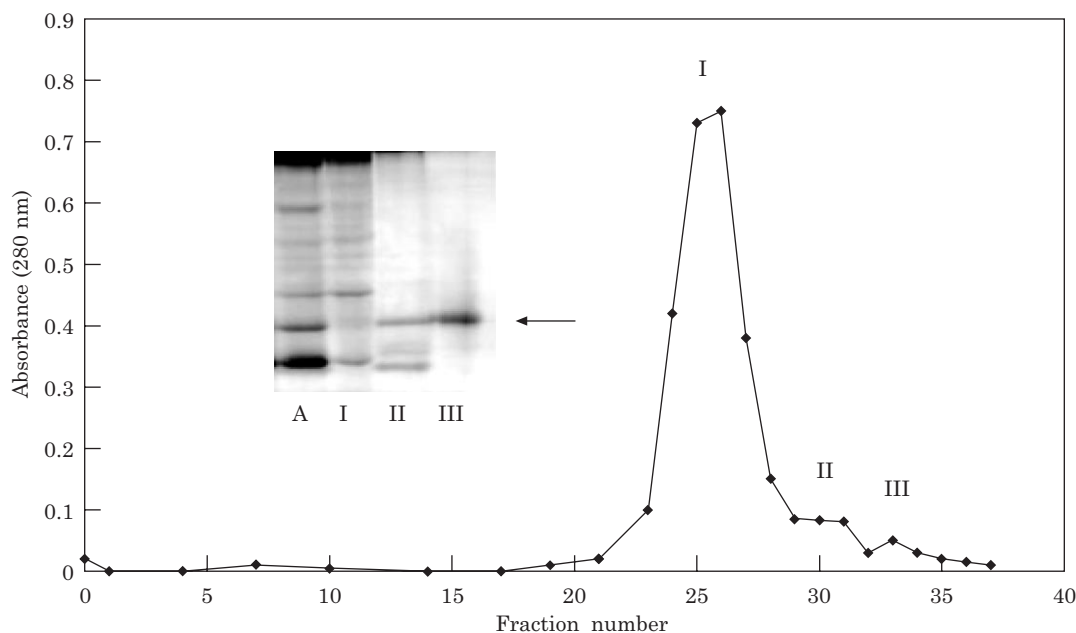


FIG. 4. Elution profile of a Sephadex G50 column on peak A from Fig. 3. The highest CF-1 specific activity was seen in fraction III (see Table 2). SDS gel electrophoresis (10% acrylamide) is shown for the starting material, peak A and for fractions I, II and III.

TABLE 3. Identification of CF-1 as variant A of SKTI by mass spectral and peptide sequence analyses

Theoretical masses for SKTI, varA fragments*	Corrected theoretical masses for SKTI, varA**	Actual mass of CF-1 fragments†	Predicted amino acid positions	Predicted polypeptide sequence
2627·10	2627·10	2627·04	77–100	GIGTISSPYRIRFIAEGHPLSL
2608·80	2608·80	2609·22	136–156	DAMDGWFRLLERVSDDDEFNN
2257·53	2356·75	2356·73	169–189	CGDIGISIDHDDGTRRLVSK
1393·55	1492·75	1492·45	157–168	LVFCPQQAEDDK‡
959·17	959·17	958·90	192–199	PLVVQFQK¶

*Theoretical masses were generated by the Peptide Mass software at ExPASy using a theoretical digestion of Swiss Prot Accession P01070 with endoproteinase Lys-C. Masses were calculated using the average mass of the occurring amino acids and giving peptide masses as $[M + H]^+$.

**The corrected theoretical mass is calculated based on alkylation of cysteines with *N*-isopropylidoacetamide (+ 99·2).

†The protein was reduced, alkylated with *N*-isopropylidoacetamide and digested with endoproteinase Lys-C.

‡The sequence of this fragment was confirmed (100% identity) by sequencing the purified polypeptide.

¶This peptide results from an anomalous cleavage of the K–P bond.

This sequence matches amino acids 157–168 in the sequence of variant A of SKTI. Variant C has also been sequenced and differs from A in substitution of E for G at amino acid 79. Since the mass spectral fragments analyzed included this position, it is clear that the purified polypeptide is variant A and not C.

DISCUSSION

In this paper the purification and characterization of CF-1, a “competency” factor required for initiation of the

phenolic polymer response in soybean cells proximal to wounded or HR dying cells are described. Through its very strong synergy with the glucan elicitor, CF-1 leads to greatly enhanced (much earlier and higher magnitude) phenolic polymer deposition in elicitor treated tissues [10]. Digestion of the final purified CF-1 with endoproteinase Lys-C and mass spectral and sequence analysis of the polypeptide fragments led to its identification as SKTI variant A (encoded by the *KTi3* gene).

There are at least 10 distinct DNA sequences in the soybean genome for the Kunitz trypsin inhibitor [19]. To date at least three of these have been confirmed to

represent functional genes, referred to as *KTi1*, *KTi2* and *KTi3*. *KTi3*, which encodes variant A (Ti^a), is the most abundantly expressed [19, 21]. The translated protein has 24 amino acids at the amino terminus and 11 amino acids at the carboxy terminus that are not present in the mature protein. The characterized KTi genes are predominantly expressed in the seed, but are also expressed in lower amounts in mature organs [19]. While the physiological function of this specific family of soybean inhibitors is unknown, there is much literature on the potential roles of trypsin and chymotrypsin inhibitors in protection of other plants against insects [28].

Protease inhibitors play central roles in a large number of regulatory processes in mammals and are a very active area of research with respect to cell activation, development and cellular response. Of particular relevance to this paper are the various redox and ion-flux activities associated with proteins carrying the soybean Kunitz domain, which are consistent with SKTI's identity as CF-1 and its association with the hypersensitive response (HR). Foremost of these reports is the fact that a protein with homology to tobacco tumor-related protein, containing a soybean Kunitz domain, was identified as one of several genes induced during and involved in the HR in tobacco [20]. This is highly consistent with the hypothesis that CF-1 is associated with the HR in soybean. Furthermore, a spinach protein with a soybean Kunitz domain has been found to function as a dehydroascorbate reductase (DHAR), a critical enzyme activity in redox regulation, when it is in the reduced (thiol) form [30]. Whether or not this activity plays a physiological role *in planta* [25], the activity is a reflection of the highly redox active sulfhydryl groups in the protein. In this reduced form, it is inactive as a trypsin inhibitor. Several other activities have also been established for other trypsin inhibitors (carrying the bovine pancreatic Kunitz domain) that could potentially relate to redox and ion channel events. For example, the BPTI Kunitz domain protein aprotinin, which stimulates skeletal muscle differentiation [32], has been identified as the first competitive inhibitor of nitric oxide synthase [31]. Nitric oxide has just recently emerged as a potential signal involved in HR and/or disease resistance in plants [2, 4, 17]. Interestingly, the BPTI Kunitz domain has also been shown to be essential to the effects of the snake venom proteins, dendrotoxins, on voltage regulated potassium channels [3, 16]. Although these various activities are consistent with the association of SKTI with HR, how they relate to its role as CF-1 currently remains unclear.

Intriguingly, the three-dimensional structure of the SKTI resembles that of interleukin-1- β and 1- α and fibroblast growth factor [26]. All of these proteins have a very unusual β -trefoil structure formed by six two-stranded hairpins. Three of these hairpins form a β -barrel and the other three form a triangular array that

caps off the barrel. An interesting parallel is that while interleukin 1 activates T lymphocyte recognition of antigens, a form of non-self recognition, CF-1 enhances response of soybean cells to the glucan elicitor from *P. sojae*, another example of non-self recognition.

Interestingly, SKTI is subject to a limited proteolytic event in soybean embryonic tissues [23]. This leads to a truncated 16 kDa form of SKTI. Although it is not known if this is involved in the activation of SKTI for CF-1 activity, it has very intriguing parallels once again to interleukin, which is proteolytically activated by interleukin converting enzyme (ICE, or caspase). Under certain conditions, the infiltration of soybean cotyledons and collection of the intercellular washing fluids lead to the release of intact SKTI and two smaller molecular weight bands of approx. molecular weights of 16 and 5 kDa (T. L. Graham *et al.*, unpublished work). The authors are currently investigating the relationship, if any, that these bands have with SKTI, its CF-1 activity and its putative association with the cell wall.

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REFERENCES

1. Ayers AR, Ebel J, Valent BS, Albersheim P. 1976. Host-pathogen interactions. X. Fractionation and biological activity of an elicitor isolated from the mycelial walls of *Phytophthora megasperma* var. *sojae*. *Plant Physiology* **57**: 760–765.
2. Delledonne M, Xia Y, Dixon RA, Lamb C. 1998. Nitric oxide functions as a signal in plant disease resistance. *Nature (US)* **394**: 585–588.
3. Dufton MJ, Harvey AL. 1998. Dendrotoxins: how does structure determine function? *Journal of Toxicology* **17**: 161–182.
4. Durner J, Wendehenne D, Kleissig DF. 1998. Defense gene induction in tobacco by nitric oxide, cyclic GMP and cyclic ADP-ribose. *Proceedings of the National Academy of Sciences, USA* **95**: 10328–10333.
5. Ebel J. 1986. Phytoalexin synthesis: the biochemical analysis of the induction process. *Annual Review of Phytopathology* **24**: 235–264.
6. Ebel J, Grisebach H. 1988. Defense strategies of soybean against the fungus *Phytophthora megasperma* f. sp. *glycinea*: a molecular analysis. *Trends in Biochemical Science* **13**: 23–27.
7. Graham TL. 1991. A rapid, high resolution HPLC profiling procedure for plant and microbial aromatic secondary metabolites. *Plant Physiology* **95**: 584–593.
8. Graham TL. 1995. Cellular biochemistry of phenylpropanoid responses of soybean to infection by *Phytophthora sojae*. In: Daniel M, Purkayastha RP, eds. *Handbook of*

- Phytoalexin Metabolism and Action*. New York, NY, U.S.A.: Marcel Dekker, 85–116.
9. **Graham MY, Graham TL**. 1991. Rapid accumulation of anionic peroxidases and phenolic polymers in soybean cotyledon tissues following treatment with *Phytophthora megasperma* f. sp. *glycinea* wall glucan. *Plant Physiology* **97**: 1445–1455.
 10. **Graham MY, Graham TL**. 1994. Wound-associated competency factors are required for the proximal cell responses of soybean to the *Phytophthora sojae* wall glucan elicitor. *Plant Physiology* **105**: 571–578.
 11. **Graham TL, Graham MY**. 1991. Glyceollin elicitors induced major but distinctly different shifts in isoflavonoid metabolism in proximal and distal soybean cell populations. *Molecular Plant–Microbe Interactions* **4**: 60–68.
 12. **Graham TL, Graham MY**. 1996. Signaling in soybean phenylpropanoid responses: dissection of primary, secondary, and conditioning effects of light, wounding, and elicitor treatments. *Plant Physiology* **110**: 1123–1133.
 13. **Graham TL, Graham MY**. 1999. Role of hypersensitive cell death in conditioning elicitation competency and defense potentiation. *Physiological and Molecular Plant Pathology* **55**: 13–20.
 14. **Graham TL, Kim JE, Graham MY**. 1990. Role of constitutive isoflavone conjugates in the accumulation of glyceollin in soybean infected with *Phytophthora megasperma*. *Molecular Plant–Microbe Interactions* **3**: 157–166.
 15. **Hahn MG, Bonhoff A, Grisebach H**. 1985. Quantitative localization of the phytoalexin glyceollin I in relation to fungal hyphae in soybean roots infected with *Phytophthora megasperma* f. sp. *glycinea*. *Plant Physiology* **77**: 591–601.
 16. **Harvey AL**. 1997. Recent studies on dendrotoxins and potassium ion channels. *General Pharmacology* **28**: 7–12.
 17. **Hausladen A, Stamler JS**. 1998. Nitric oxide in plant immunity. *Proceedings of the National Academy of Sciences, USA* **95**: 10345–10347.
 18. **Ingham JL**. 1982. Phytoalexins from the Leguminosae. In: Bailey JA, Mansfield JW, eds. *Phytoalexins*. New York, NY, U.S.A.: Wiley, 21–80.
 19. **Jofuke KD, Goldberg RB**. 1989. Kunitz trypsin inhibitor genes are differently expressed during soybean life cycle and in transformed tobacco plants. *Plant Cell* **1**: 1079–1093.
 20. **Karrer EE, Beachy RN, Holt CA**. 1998. Cloning of tobacco genes that elicit the hypersensitive response. *Plant Molecular Biology* **36**: 681–690.
 21. **Kim S-H, Hara S, Hase S, Ikenaka T, Kitamura K, Kaizuma N**. 1985. Comparative study on amino acid sequences of Kunitz-type soybean trypsin inhibitors, Ti^a, Ti^b, and Ti^c. *Journal of Biochemistry* **98**: 435–448.
 22. **Laemmli UK**. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.
 23. **Mcgrain AK, Chen JC, Wilson KA, Tan Wilson AL**. 1992. Proteases catalyzing processing and degradation of Kunitz soybean trypsin inhibitor during seed maturation. *Phytochemistry* **31**: 421–426.
 24. **Mohr PG, Cahill DM**. 2001. Relative roles of glyceollin, lignin and the hypersensitive response and the influence of ABA in compatible and incompatible interactions with *Phytophthora sojae*. *Physiological and Molecular Plant Pathology* **58**: 31–41.
 25. **Morell S, Follmann H, DeTullio M, Haberlein I**. 1997. Dehydroascorbate and dehydroascorbate reductase are phantom indicators of oxidative stress in plants. *FEBS Letters* **414**: 567–570.
 26. **Murzin AG, Lesk AM, Chothia C**. 1992. Beta-trefoil patterns of structure and sequence in the Kunitz inhibitors, interleukin 1-beta and 1-alpha and fibroblast growth factors. *Journal of Molecular Biology* **223**: 531–544.
 27. **Rivera-Vargas LI, Schmitthener AF, Graham TL**. 1993. Soybean flavonoid effects on and metabolism by *Phytophthora sojae*. *Phytochemistry* **32**: 851–857.
 28. **Ryan CA**. 1990. Protease inhibitors in plants—genes for improving defenses against insects and pathogens. *Annual Review of Phytopathology* **28**: 425–449.
 29. **Schmitthener AF**. 1985. Problems and progress in control of *Phytophthora* root rot of soybeans. *Plant Disease* **69**: 362–368.
 30. **Truemper S, Follman H, Haerberlein I**. 1994. A novel dehydroascorbate reductase from spinach chloroplasts homologous to plant trypsin inhibitor. *FEBS Letters* **352**: 159–162.
 31. **Venturini G, Colasanti M, Ascenzi P**. 1998. Aprotinin, the first competitive inhibitor of NOS activity. *Biochemistry and Biophysics Research Communication* **249**: 263–265.
 32. **Wells JM, Strickland S**. 1994. Aprotinin, a Kunitz-type protease inhibitor, stimulates skeletal muscle differentiation. *Development* **120**: 3639–3647.
 33. **Yoshikawa M, Yamauchi K, Masago H**. 1978. Glyceollin: its role in restricting fungal growth in resistant soybean hypocotyls infected with *Phytophthora megasperma* var. *sojae*. *Physiological Plant Pathology* **12**: 73–82.