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Isolation and purity determination of a glycoprotein elicitor from wheat stem rust by medium-pressure liquid chromatography

B. BEISSMANN* and H. J. REISENER
RWTH Aachen, Institut für Biologie III, Worringerweg, D-5100 Aachen (F.R.G.)

ABSTRACT

Previously the isolation of a single glycoprotein (pgt-elicitor) from cell walls of the phytopathogenic fungus *Puccinia graminis* f.sp. *tritici* Erics. & Henn., which elicits defence reactions in wheat leaves was described. The apparent molecular mass of this compound, isolated via concanavalin A affinity chromatography and anion-exchange chromatography, was 67 000 as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis. A scaled-up procedure for purifying larger amounts of fungal elicitor based on anion-exchange fast protein liquid chromatography (FPLC) with Q-Sepharose Fast Flow medium has now been developed. The yield of pure elicitor was further increased by the use of the volatile buffer ammonium carbonate for eluting the bound glycoproteins. Analytical size-exclusion FPLC supplemented gel electrophoretic results by quantifying small amounts of inactive components contaminating the most active elicitor fraction. Further, purity determination by FPLC provided evidence of well defined complex formation leading to co-migration of elicitor-associated glycoproteins during anion-exchange chromatography. The glycoprotein complexes were separated during size-exclusion FPLC at lowered pH (100 m*M* acetic acid). Thus, size exclusion FPLC resulted in final purification of the pgt-elicitor.

INTRODUCTION

Active disease resistance in plants involves inducible defence mechanisms, such as accumulation of chemical (phytoalexin antibiotics) [1,2] and structural barriers (lignin-like material or callose) [3] or increasing activities of cell wall-degrading enzymes (chitinase and β-1–3-glucanase) [4], which block infection by a potential pathogen. Defence reactions as induced by pathogen invasion can be simulated by molecules termed elicitors. A wide range of molecules of fungal or bacterial origin such as fatty acids, polysaccharides, glycoproteins and enzymes [5] have been described as elicitors in different host–parasite interactions.

Wheat plants highly resistant to the attack from the fungal pathogen *Puccinia graminis* f.sp. *tritici* Erics & Henn., which causes wheat stem rust disease, are characterized by the so-called hypersensitivity reaction. This reaction consists in rapid lignification and subsequent cell death in infected leaf tissue, thus preventing further fungal growth [6,7]. Apparently, phytoalexins are not involved in the hypersensitivity reaction of wheat plants.

A glycoprotein fraction isolated from germ tubes of *Puccinia graminis* f. sp. *tritici-uredospores* elicits symptoms in wheat leaves similar to those observed after rust

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infection [8–10]. Fractionation of this material by affinity chromatography using concanavalin A (Con A)-Sepharose, followed by anion-exchange fast protein liquid chromatography (FPLC), yielded a highly pure Con A binding glycoprotein elicitor (pgt-elicitor) with a relative molecular mass of about 67 000 dalton [11]. The active site of the elicitor consists of carbohydrate structures [11] and is mainly composed of galactose (50%) and mannose (47%). The inactive peptide portion represents 7% of the glycoprotein only. Purification of larger amounts of this glycoprotein has been a prerequisite for the evaluation of structural requirements for elicitor activity in more detail. The aim of this work was therefore to scale up the purification of the pgt-elicitor. In addition, the advantages and applications of size-exclusion FPLC *versus* sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) for purity determination of glycoproteins are discussed.

EXPERIMENTAL

Materials

All chemicals were of analytical-reagent grade or higher quality, if not stated otherwise.

Nitrocellulose membranes of 0.45 μm pore size were purchased from Schleicher & Schüll (Dassel, F.R.G.). Acetic anhydride, betaine, Con A, fish gelatin, methyl α-D-mannopyranoside, protein standards, sodium dodecyl sulfate, taurine and trifluoroacetic acid were supplied by Sigma (F.R.G.), anthrone, acetic acid, ammonium carbonate, bisacrylamide, ethanol, glutardialdehyde, glycerol, hydrochloric acid, phosphoric acid, polyacrylamide, silver nitrate, sodium carbonate, sodium chloride and tris(hydroxymethyl)aminomethane (Tris) from Merck (Darmstadt, F.R.G.) and Ammonium peroxodisulphate and horseradish peroxidase from Serva (Heidelberg, F.R.G.).

The FPLC equipment consisted of a GP-250 gradient programmer, two P 500 pumps, a Uvicord II Model 2238, a Model 2210 two-channel recorder, and a Frac-100 fraction collector (Pharmacia–LKB, Uppsala, Sweden). All chromatographic media including prepacked columns of Mono Q HR 5/5, Superose 12 HR 10/30 and the PD 10 desalting column and laboratory-filled columns of Q-Sepharose Fast Flow HR 10/10 and Con A-Sepharose 4 B were obtained from Pharmacia–LKB.

Extraction of glycoproteins

Elicitor-active glycoproteins were solubilized from germ tube walls of *Puccinia graminis* using a published method [8,9]. The protein concentration of this crude elicitor preparation (CEP) was determined according to Bradford [12]; the carbohydrate content was determined by the anthrone method [13].

Assays for elicitor activity

Samples to be tested for elicitor activity (50 μl) were injected into the intercellular spaces of five wheat primary leaves using a hypodermic syringe [14]. Growth conditions for wheat have been described previously [14]. Infiltrated areas of the primary leaves were cut off 24 h later, frozen in liquid nitrogen and tested for phenylalanine–ammonia–lyase (PAL) activity [15]. Dose–response curves were measured to determine the specific elicitor activity, which was defined as the specific

enzyme activity induced per gram of elicitor carbohydrates (μkat kg^{-1} protein g^{-1} carbohydrate) [11]. The extent of lignification was assessed microscopically by observing yellow autofluorescence [11] or positive phloroglucinol staining [16] of affected cells.

Affinity chromatography with Con A Sepharose

A column of immobilized Con A (Con A-Sepharose 4 B, 5 × 1 cm I.D.) was equilibrated with 20 m*M* Tris–HCl (pH 7.4) containing 0.5 *M* NaCl and 1 m*M* each of $MgCl_2$, $MnCl_2$ and $CaCl_2$ (starting buffer). The CEP (3–5 mg glucose equivalents), dissolved in 3 ml of the same buffer, was applied to the column. Con A-Sepharose was eluted successively with 50 ml of starting buffer, then with 0.2 *M* methyl α-D-mannopyranoside in starting buffer. The absorbance was recorded at 280 nm. Fractions of 2 ml were pooled. All buffers contained 0.1% *n*-butanol to prevent microbial contamination. Con A-binding glycoproteins were identified in a fast dot blot assay. Samples of 10 μl were dotted on a nitrocellulose sheet and stained as described [11].

Gel electrophoresis, blotting and staining procedures

Glycoproteins were separated by SDS-PAGE using 16-cm 12.5% polyacrylamide gels and the Laemmli buffer system [17]. For lectin staining, proteins were electroblotted to nitrocellulose [18] using a transblot cell (Bio-Rad Labs., Munich, F.R.G.). The transfer buffer contained 25 m*M* Tris and 192 m*M* glycine (pH 8.3) with 20% methanol (v/v), and transfer was carried out at 400 mA for 2 h with cooling to 4°C. Blots were first incubated in Tris-buffered saline [50 m*M* Tris–HCl–200 m*M* NaCl (pH 7.9)] supplemented with 0.45% of fish gelatin. Con A-binding glycoproteins were detected by Con A–peroxidase staining as described [19].

For more sensitive detection, glycoprotein samples were run in 8–25% gradient gels using a PhastGel separation system (Pharmacia–LKB). Proteins were stained with silver [20].

Gas chromatographic determination of carbohydrate content

Monosaccharides of the carbohydrate part were released by acid hydrolysis of the pgt-elicitor. Alditol acetate derivatives of the products of hydrolysis were prepared by the method of Jones and Albersheim [21] and separated by gas chromatography as described [11].

Anion-exchange FPLC with Mono Q

The separation of glycoproteins was carried out by FPLC with a prepacked Mono Q HR 5/5 anion-exchange column. Elution was performed at a flow-rate of 1 ml min^{-1} using two buffers. Buffer A contained 25 m*M* Tris–HCl (pH 8.5) with 4% (w/v) of betaine or tautine to minimize ionic interactions of molecules [22]. Buffer B contained 1 *M* NaCl in the same buffer as A. Eight minutes after injection of the sample, the ratio of buffer B to A was increased with a linear gradient to 17% in 16 min. Strongly binding (glyco)proteins were finally eluted with 100% buffer B. The fractions were desalted on prepacked PD 10 gel filtration columns.

Anion-exchange FPLC with Q-Sepharose Fast Flow

Crude elicitor preparation (50 mg in 50 ml) was injected onto a laboratory-packed Q-Sepharose Fast Flow HR 10/10 column equilibrated with 20 m*M* $(NH_4)_2CO_3$ (pH 9.1). The column was washed for 60 min at a flow-rate of 1 ml min^{-1} and eluted with a 220-min gradient from 20 to 200 m*M* $(NH_4)_2CO_3$. Fractions were collected from different runs. Desalting could easily be achieved by lyophilizing the volatile buffer components.

In order to regenerate the Q-Sepharose Fast Flow gel, the column was washed with 50 ml of distilled water (flow-rate 1 ml min^{-1}) followed by a 10-ml wash with 2 *M* NaOH (flow-rate 0.5 ml min^{-1}) and a 10 ml wash with 1 *M* NaCl (flow-rate 0.5 ml min^{-1}). Finally, the column was re-equilibrated with 10 m*M* $(NH_4)_2CO_3$.

Size-exclusion FPLC

Fractionation of glycoproteins by size-exclusion FPLC was conducted on Superose 12 HR 10/30. The column, equilibrated with 100 m*M* acetic acid, was calibrated with the standard proteins aldolase (molecular weight, M_r 146 000), bovine serum albumin (BSA) (M_r 66 000) and carbonic anhydrase (M_r 29 000).

Samples of 200 μl containing up to 60 μg of glycoprotein were applied to the column per run. Size-exclusion FPLC was performed at a flow-rate of 0.3 ml min^{-1}.

RESULTS AND DISCUSSION

Affinity chromatography

Many proteins of the crude cell wall elicitor preparation (CEP) eluted unbound from the Con A-Sepharose column as indicated by an increased absorbance at 280 nm, but this material was inactive in bioassays for elicitor activity. Active molecules were displaced by 0.2 *M* methyl α-D-mannopyranoside in Con A buffer. Negative staining of the unbound material in a spot dot test after Con A–peroxidase overlay and chloronaphthol–hydrogen peroxide addition confirmed the binding activity of the column-immobilized lectin. Gel electrophoresis under denaturing conditions of the Con A binding fraction and staining revealed several glycoprotein bands ranging in molecular weight from 20 000 to 95 000 dalton [11].

For further purification, fractions were desalted by extensive dialysis against distilled water or repeated gel filtration (up to three times) on PD 10 desalting columns. Gas chromatographic carbohydrate determination showed that a single gel filtration step was ineffective for complete removal of methylmannosides. Difficulties in separating phytotoxic glycopeptides and methylmannosides were also described by Lazarovits *et al.* [23]. We assume that aggregation between methyl α-D-mannopyranoside and the glycoprotein elicitor interferes with the separation.

Affinity chromatography followed by desalting procedures resulted in a considerable loss of glycoproteins, amounting to more than 50% of the starting material.

Anion-exchange FPLC with Mono Q

Con A binding glycoproteins were further separated by anion-exchange FPLC on a Mono Q HR 5/5 column. Elution of bound material with NaCl resulted in four main peaks, which were sharply baseline separated (Fig. 1). Peaks C and D both contained molecules with high elicitor activity, whereas the unbound material was inactive and the material of peaks A and B exhibited only low activity.

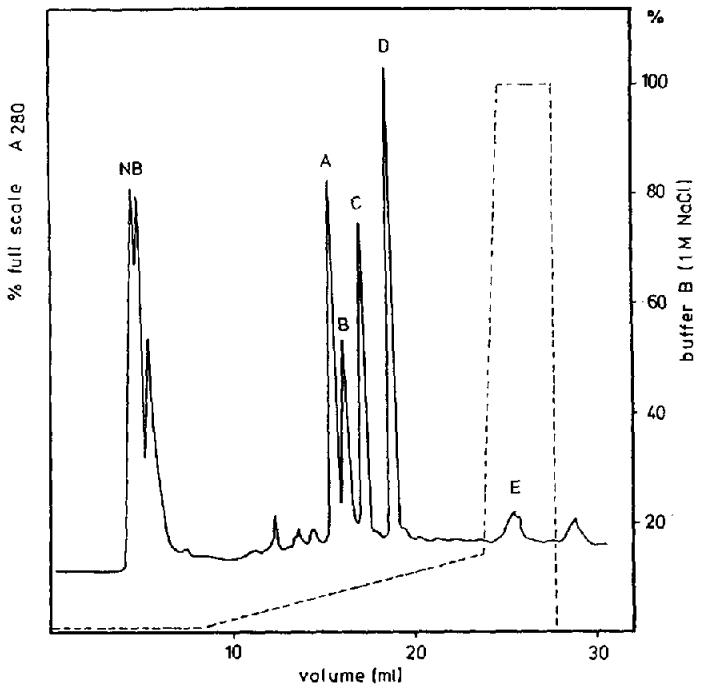

Fig. 1. Anion-exchange FPLC of the CEP isolated from germ tube walls of *Puccinia graminis* uredosporelings. 6 mg of CEP in 10 ml of Tris–HCl buffer (pH 8.5) containing 4% of betaine were applied to a Mono Q HR 5/5 column. Elution was performed at a flow-rate of 1 ml min^{-1} with a linear gradient of 0–17 m*M* NaCl. Peak C exhibited very high elicitor activity and was referred to as pgt-elicitor.

SDS-PAGE showed the most active fraction C to consist of a single glycoprotein with a molecular weight of 67 000. This glycoprotein also appeared in fraction D and to a minor extent in fractions A and B. The 67-kilodalton glycoprotein was the only active part of all fractions, as proved by re-extraction from polyacrylamide gel [11]. Fractions A and B additionally contained at least four low-molecular-weight and one high-molecular-weight glycoprotein. We also detected some faintly stained indistinct bands of low-molecular-weight components in fractions C and D. This effect was not observed in previous studies [11] and may be due to a slightly changed quality of elicitor preparation, as indicated by the fact that now all binding (glyco)proteins eluted at lower NaCl concentration.

The finding that all fractions contained the active glycoprotein, although the peaks were baseline separated, suggested that glycopeptide interaction led to stable complexes co-migrating during anion-exchange chromatography. However, there is no simple subunit relationship, indicating a more complex association of different glycoproteins. The assumption of complex formation is confirmed by the finding of smaller elicitor-active glycoproteins only after gel filtration under denaturing conditions as described previously [11]. In the absence of SDS, the active glycoproteins formed aggregates larger than 130 000 dalton [11]. Complex formation of glycopeptides was also detected on fractionating phytotoxic glycopeptides of *Stemphylium botryosum* [24].

CEP was directly subjected to FPLC-associated Mono Q anion-exchange chromatography. The elution profile and the distribution and gel electrophoretic behaviour of the active fractions showed no difference to the anion-exchange FPLC of prepurified glycoproteins (Fig. 1). However, after performing 3–5 runs each with 6 mg of CEP dissolved in 10 ml of separation buffer, the back-pressure of the column increased considerably and the elution quality decreased, yielding broader peaks. Neither washing with salts, acids and bases as described [25] nor changing prefilters resulted in regeneration of the column. The separation capacity could only be restored by removal of the Mono Q gel, incubating it in 50% (v/v) methanolic 1 *M* hydrochloric acid for 1 h at room temperature and finally refilling the column. The procedure is time consuming and may shorten the lifetime of the column.

Semi-preparative anion-exchange FPLC

The aim of isolating larger amounts of glycoprotein elicitor was achieved by performing anion-exchange FPLC with Q-Sepharose Fast Flow medium packed in an HR 10/10 column. Without any kind of prepurification the chromatographic separation of CEP on this column led to an elution profile comparable to that recorded from FPLC with Mono Q (Fig. 2). Fraction C again contained the most active material, as shown by measuring dose–response curves of PAL-inducing activity. Interestingly, after SDS-PAGE all active fractions showed the same glycoprotein pattern, with the exception of fraction C, which contained large amounts of the pgt-elicitor together with a small portion of smaller glycopeptides (Fig. 3). The yield of

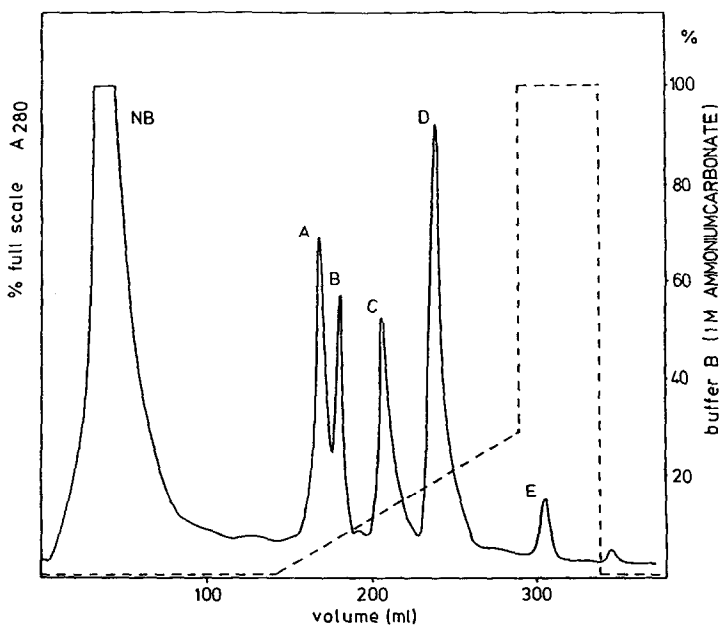

Fig. 2. Anion-exchange FPLC of CEP isolated from mycelial walls of *Puccinia graminis* uredosporelings. 12 mg of CEP in 20 ml of $(NH_4)_2CO_3$ buffer were applied to a Q-Sepharose Fast Flow HR 10/10 column. Elution was performed at a flow-rate of 1 ml min^{-1} with a linear gradient of 20–200 m*M* $(NH_4)_2CO_3$.

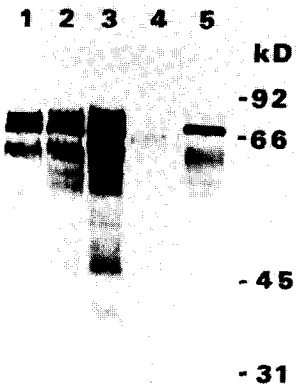

Fig. 3. Electroblot of glycoproteins from 12.5% SDS-PAGE of elicitor-active fractions (A, B, C, D) obtained by anion-exchange FPLC on a Mono Q HR 5/5 column (Fig. 2). Glycoprotein bands were stained with Con A–peroxidase. 0.5 μg glucose equivalents of each fraction were applied to the gel. Lanes: 1 = fraction D; 2 = fraction A; 3 = fraction B; 4 = fraction E; 5 = fraction C. kD = kilodalton.

highly active elicitor fraction obtained by the scaled-up procedure was further increased by using $(NH_4)_2CO_3$ as eluent. Without any loss of active material due to desalting procedures, the volatile buffer compound was removed by freeze-drying. More than 1.5 mg of purified pgt-elicitor were obtained by purifying CEP containing 18 mg glucose equivalents.

Mono Q versus *Q-Sepharose FPLC*

Rechromatography of fraction C separated by Q-Sepharose Fast Flow medium on the Mono Q column resulted in a large peak corresponding to the pgt-elicitor and some very small peaks corresponding to fractions A, B and D (data not shown). Material taken from the sample of fraction C after FPLC with the Mono Q column was reapplied to the Monio Q column. During the following separation only one peak, C, was recorded. Obviously, FPLC on the Mono Q column yielded elicitor of higher purity than FPLC on a Q-Sepharose Fast Flow column.

Separation of the carbohydrate content by gas chromatography pointed to an additional advantage of the new semi-preparative purification method. The monosaccharide composition of the most active fraction C consisted of 22% mannose, 77% galactose and traces of N-acetylglucosamine, but less than 1% of glucose was detected. Thus, it became evident that the 3% of glucose found in fraction C after the two-step purification [11] was a contaminant from polyglucose separation matrices, *e.g.*, the Con A Sepharose or the PD 10 desalting columns. The enrichment of galactose content from 47% to 77% achieved by Q-Sepharose Fast Flow-FPLC indicates another difference in the purification.

Complex formation of the pgt-elicitor

CEP was separated by size-exclusion FPLC with Superose 12 into seven well resolved peaks corresponding to molecular masses from 300 to 1 kilodalton (Fig. 4). The amount of each eluting (glyco)protein could be calculated from the peak area and

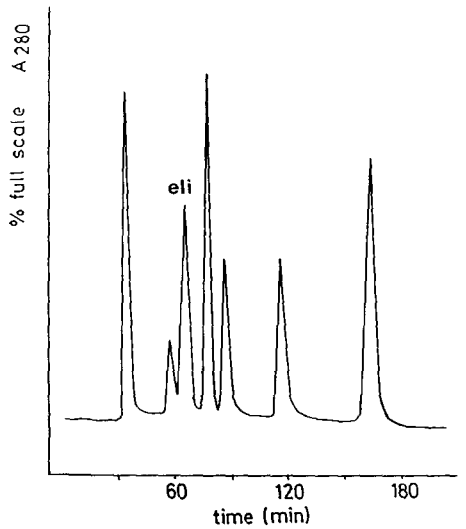

Fig. 4. Analytical size-exclusion FPLC of CEP on a Sepharose 12 HR HR10/30 column. For application, CEP was dissolved in 200 μl of 100 m*M* acetic acid. Elution was performed at a flow-rate of 0.3 ml min^{-1} with 100 m*M* acetic acid. Eli = pgt-elicitor.

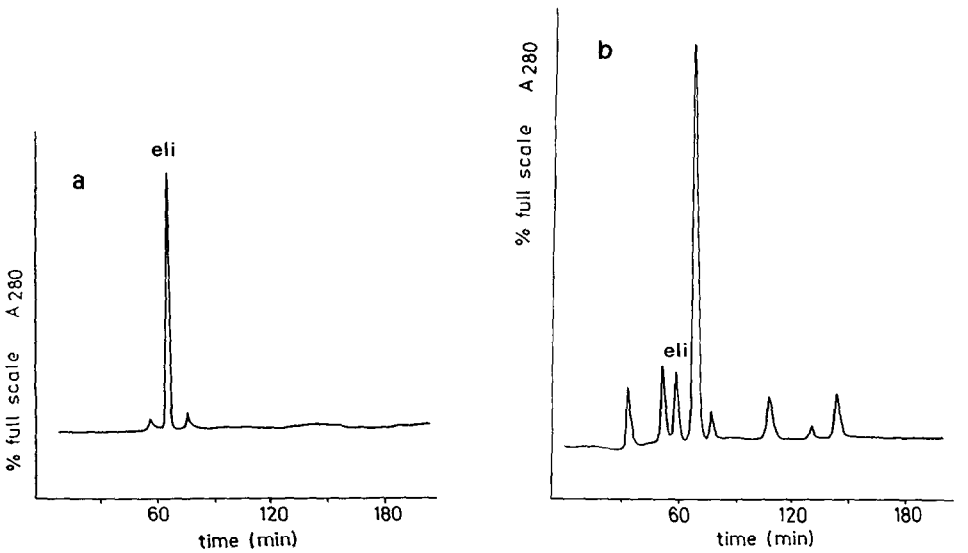

Fig. 5. (a) Analytical size-exclusion FPLC of fraction C exhibiting high elicitor activity, which was isolated by anion-exchange FPLC on a Mono Q HR 5/5 column (Fig. 2). 60 μg of the glycoprotein fraction A were dissolved in 200 μl of 100 m*M* acetic acid and applied to a Superose 12 HR 10/30 column. Elution was performed at a flow-rate of 0.3 ml min^{-1} with 100 m*M* acetic acid. (b) Analytical size-exclusion FPLC of fraction B exhibiting low biological activity, which was isolated by anion-exchange FPLC on a Mono Q HR 5/5 column (Fig. 1). A 60-μg amount of the glycoprotein fraction B was dissolved in 200 μl of 100 m*M* acetic acid and applied to a Superose HR 10/30 column. Elution was performed at a flow-rate of 0.3 ml min^{-1} with 100 m*M* acetic acid. Eli = pgt-elicitor.

shown to be highly reproducible but varying with each elicitor preparation. In order to prove whether the high-molecular-mass compounds were due to aggregation of glycoproteins, the material of CEP higher than 300 000 dalton was sampled over several runs and rechromatographed on a Superose 12 column eluted with 100 m*M* acetic acid. As expected, the complexes dissociated, yielding four new peaks including one peak corresponding to the pgt-elicitor (data not shown). Hence, the four glycoproteins with molecular weights from about 150 000–20 000 dalton obviously interact in a defined manner underlying a complex dissociation equilibrium.

Determination of purity

The results clearly demonstrate a correlation between increasing purity and specific elicitor activity during anion-exchange chromatography. The fractionation of CEP on Mono Q yielded a sharply separated peak C, indicating high purity of the pgt-elicitor (Fig. 1). This result did not correlate with gel electrophoretic analysis, indicating contamination by complex-forming glycoproteins as described above. Therefore, we additionally checked the purity of the pgt-elicitor isolated via anion-exchange FPLC by size-exclusion FPLC.

Under the conditions described above, the Mono Q anion-exchange FPLC fractions A, B, C and D were subjected to size-exclusion FPLC on a prepacked Superose 12 column. Interestingly, the elicitor activity of the fractions was strongly correlated with the peak height of a glycoprotein eluting at the same time as BSA (M_r 66 000). This glycoprotein was identical with the major component of fraction C and was therefore identified as pgt-elicitor (Fig. 5a). Fraction C additionally contained two minor (glyco)protein peaks with relative molecular masses of *ca.* 150 000 and 45 000 dalton. The latter might correspond to the broad band of low molecular weight glycoprotein(s) faintly stained below the pgt-elicitor after SDS-PAGE (Fig. 3, lane 5).

The FPLC Mono Q fractions A and B showed only small peaks corresponding to the pgt-elicitor, but additionally some larger peaks of other (glyco)proteins (Fig. 5b). These results confirm the conclusion that all active fractions share the pgt-elicitor as the only active compound. The elution profiles of all anion-exchange fractions during size-exclusion FPLC were highly reproducible. Therefore, we assume that specific complex formation occurs between the pgt-elicitor and other glycoproteins.

For evaluation of glycoprotein purity, size-exclusion FPLC at low pH was evidently superior to SDS-PAGE, as the relative amounts of different glycoproteins associated with the pgt-elicitor could be quantified. Interestingly, molecular weight determination of the pgt-elicitor by SDS-PAGE and size-exclusion FPLC led to nearly identical results. Often, the molecular size of glycoproteins is underestimated by SDS-PAGE and overestimated by size-exclusion chromatography [26].

Final purification of the pgt-elicitor

Pgt-elicitor was purified by size-exclusion FPLC on the analytical Superose 12 column. When subjected to PhastSystem SDS-PAGE, only a single protein (M_r 67 000) was stained by ultrasensitive silver staining (Fig. 6). In contrast to both kinds of anion-exchange FPLC, size-exclusion FPLC obviously resulted in maximum purification of the pgt-elicitor. However, as described above, pgt-elicitor formed large amounts of high-molecular-mass complexes which remained partly unresolved during size-exclusion FPLC, thus probably contributing to a considerable loss of active

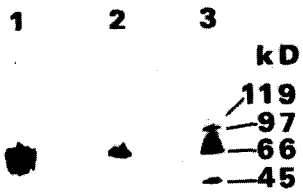

Fig. 6. SDS-PAGE of pgt-elicitor isolated via anion-exchange and size-exclusion MPLC. The (glyco)-protein fractions were separated by the PhastSystem in a gradient gel of 8–25% polyacrylamide and silver stained. Lanes: 1 = pgt-elicitor purified by Mono Q anion-exchange MPLC; 2 = pgt-elicitor purified by Superose 12 size-exclusion MPLC; 3 = molecular weight markers (kD = kilodalton).

material. Therefore, we do not recommend the scaling up of size-exclusion FPLC as a semi-preparative procedure for optimized elicitor purification.

Alternatively, elicitor purification by anion-exchange FPLC with Q-Sepharose Fast Flow medium presents an abundant source of highly active material. Further studies will concern the cleavage of the glycoprotein and the isolation of active molecular structures, thus leading to a final purification of elicitor-active components. As only the pgt-elicitor exhibits elicitor activity [11], slight contamination of inactive material will not affect studies on the physiological effects of the elicitor. Greater amounts of highly active material will also be helpful in continuing studies of the characterization of elicitor binding sites as putative receptor molecules in wheat plants [27].

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