Investigations into the molecular size and shape of tomato extensin

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The molecular characteristics of soluble extensin from tomato have been investigated. An apparent molecular mass greater than 240 kDa has been previously observed with the shape-dependent method of gel-filtration chromatography [Brownleader and Dey (1993) *Planta* (Berlin) **191**, 457–469]. Tomato extensin is a heavily glycosylated protein that does not migrate into SDS/polyacrylamide gels. This shape-dependent behaviour raises doubts about agreement between the observed apparent mass and the absolute value. The molecular mass measured with matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI–TOF MS) was 72.3 kDa, with no evidence of any other species except a doubly charged ion. The sample was therefore considered to be monodisperse under the conditions used. Electron microscopy of soluble extensin showed the presence of particles 40–50 nm in length and 2.0–2.5 nm in width. A minority of these particles showed a central ‘kink’. A number of smaller and generally wider particles (20 nm × 2–4 nm)

were considered to be folded monomers and larger particles were thought to be dimers. Sedimentation analysis showed that extensin exists in a rapid monomer–dimer equilibrium in the concentration range and buffer used. Sedimentation equilibrium data gave a K_d of 8.5 μ M and sedimentation velocity data generated a K_d between 1 and 10 μ M. The concentration dependence of the measured sedimentation coefficient was used, together with hydrodynamic bead modelling, to define plausible shapes for monomer and dimer. This suggests that monomeric extensin is an elongated rod of length 40 nm and width 2 nm, which forms staggered dimers of average length 50 nm and width 3 nm. Extensin is an integral component of the primary cell wall. The physical characteristics (size, shape and form) of the rod-like extensin have been evaluated in this paper so that the role that extensin plays in primary cell wall architecture and during plant disease resistance can be more fully understood.

INTRODUCTION

Plants activate a large array of inducible defence responses as a result of microbial challenge, injury or other forms of stress. At the cell wall level, these responses include an elevated peroxidase activity and enhanced insolubilization of hydroxyproline-rich glycoproteins (HRGPs). The overall levels of HRGP in plant tissues have been shown to increase as a result of fungal and bacterial infection, or treatment with elicitors obtained from the pathogen [1–6]. Cooper and Varner [6] demonstrated that cell wall HRGP, extensin, is insolubilized in the carrot cell wall by a peroxidase/hydroperoxide-mediated cross-linking process. Esquerré-Tugaye et al. [2] suggested that, in musk melon, HRGP accumulation in the cell wall of *Colletotrichum lagenarium*-infected plants was a defence mechanism against the pathogen. The speed of cross-linking (insolubilizing) HRGPs in bean and soybean cells after incubation with fungal elicitors suggested a rapid defence mechanism, preceding gene expression, where insolubilized HRGPs are believed to strengthen the cell wall barrier against an invading pathogen [7]. We have isolated and purified extensin [8] and a cross-linking peroxidase [9] to homogeneity from suspension-cultured tomato cells and demonstrated the cross-linking of extensin *in vitro* by a sizing chromatography assay. We now report some of the properties of the soluble extensin.

Stuart and Varner [10] demonstrated that soluble extensin from carrot did not enter SDS/polyacrylamide gels or SDS/8 M

urea/polyacrylamide gels. However, the authors successfully performed electrophoresis by using acidic (pH 3.0) 8 M urea/polyacrylamide gels. We found that these gels were relatively difficult to prepare, and do not give particularly clear results. The protein bands were also difficult to stain with Coomassie Brilliant Blue. Difficulties with electrophoresis and staining were attributed to extensin's high content of carbohydrate, hydroxyproline and basic amino acids. Buoyant density measurements showed that carrot extensin comprises about 50% carbohydrate [10,11]. Stafstrom and Staehelin [12] purified two ionic forms of extensin, extensin-1 and extensin-2, from carrot. By SDS/PAGE, the molecular mass of extensin-2 was found to be 145 kDa whereas extensin-1 hardly migrated into the gel. Smith et al. [13] demonstrated that the two ionic forms of tomato extensin also migrated poorly on SDS/polyacrylamide gels and did not stain well with Coomassie Blue. However, the deglycosylated samples of the two ionic forms migrated well on an SDS/polyacrylamide gel and banded as proteins with mobilities of 55 and 53.5 kDa. Accounting for the carbohydrate content, the molecular mass of the native tomato extensin would be approx. 100 kDa. The role of carbohydrate in extensin is believed to be in maintaining an extended conformation of the molecule [14].

We have resolved two molecular forms of soluble extensin from suspension-cultured tomato cells, one form eluting in the void volume on a Superose-12 gel-filtration column and the other eluting just ahead of catalase (240 kDa), which is at the border of the linear range of the calibration curve of the column.

Abbreviations used: HRGP; hydroxyproline-rich glycoprotein; MALDI–TOF MS, matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry.

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This led us to suggest a molecular mass in excess of 240–300 kDa for this extensin [8]. We further showed that native extensin did not migrate into an SDS/polyacrylamide gel [15]. A knowledge of the precise molecular mass and shape of extensin reported in this paper is important in order to understand the role of extensin in cell wall architecture and also its significance in plant disease resistance when deposited in the wall by the cross-linking mechanism.

MATERIALS AND METHODS

Materials

The suspension culture of tomato cells (hybrid of *Lycopersicon esculentum* and *L. peruvianum*) was a gift from Professor Stephen Fry (Institute of Cell and Molecular Biology, University of Edinburgh, Edinburgh, Scotland, U.K.). All other chemicals, except CM-52 cellulose resin (Whatman, Maidstone, U.K.) and Sephacryl S-200 resin (Pharmacia, St. Albans, U.K.), were purchased from Sigma (Poole, Dorset, U.K.).

Growth conditions of suspension culture

Suspension-cultured tomato cells were grown in Murashige and Skoog-based tissue culture medium (basal salt mixture; Sigma) [16] containing 20 g/l glucose and a supplement solution consisting of 100 mg/l *myo*-inositol, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine/HCl, 0.1 mg/l thiamine/HCl, 2 mg/l glycine, 10 mg/l indole-3-acetic acid and 0.5 mg/l kinetin. Conical flasks (500 ml capacity) containing 200 ml of sterilized medium were subcultured with 7-day-old suspension cells and incubated on a gyratory shaker at 110 rev./min under continuous fluorescent lighting for 7 days at 21 °C.

Salt elution of soluble extensin and extensin peroxidase

Typically, 350 g wet weight of tomato cells were washed extensively in a Buchner funnel with 5 litres of pre-cooled distilled water. Calcium chloride solution (200 ml, 50 mM, made to pH 3.0 with HCl) was poured on the water-washed cell pad and allowed to percolate without suction for 5 min. Gentle suction was then applied and all salt-eluted protein was collected in the Buchner flask. This elution was repeated and all the eluted protein was pooled. The solution was subsequently dialysed against 5 litres of water (made to pH 3.0 with HCl) overnight, the low pH being unfavourable to peroxidase action and therefore preventing cross-linking if any H₂O₂ or organic hydroperoxides were present. Dialysis was continued for a further 3 h in distilled water.

Purification of soluble extensin and extensin peroxidase

The dialysed salt-eluted protein was concentrated by passing it through a CM-cellulose cation exchange column (3 cm × 3.5 cm) equilibrated with degassed 20 mM McIlvaine's buffer (Na₂HPO₄/citric acid), pH 5.5 [17]. The bound proteins were eluted with the same buffer but containing 0.5 M NaCl. The protein-containing fractions were concentrated to 5 ml by ultra-filtration (10 kDa Omega membrane; Filtron, Northborough, MA, U.S.A.).

The concentrated sample was then applied to a Sephacryl S-200 gel-filtration column (90 cm × 2.5 cm) equilibrated with degassed 0.1 M sodium acetate buffer, pH 5.0, containing 0.1 M NaCl and 0.02 % NaN₃. Protein fractions were monitored at 280 nm and the haem of the peroxidase was monitored at 405 nm. Extensin was purified to homogeneity as described earlier [8] and was used for further experiments.

Matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI–TOF MS)

Spectra were obtained on a Fisons Instruments VG Analytical ToFSpec MALDI–TOF mass spectrometer. The extensin samples (0.2 mg/ml) were diluted 1:10 and then run from a 50:50 (v/v) mixture with the sinapinic acid matrix. The sample matrix was dried on a stainless steel target and ionized by irradiation with UV from a nitrogen laser at 337 nm. The data from 50 laser shots were acquired and averaged to produce the spectrum shown. BSA (66430 Da) was used as the external calibrant.

Analysis of native extensin by electron microscopy

Aliquots (20 µl) of 50 % (v/v) glycerol solutions of extensin at a final concentration of approx. 0.2 mg/ml were sprayed from micropipette tips on a freshly cleaved mica surface, using a specially adapted artist's spray brush with high-pressure nitrogen as a propellant. The mica surface was then unidirectionally shadowed with thermally evaporated platinum at a 5 ° angle. After backing with a thin layer of carbon, the replica was floated off on water, collected on 200 mesh copper grids and examined in a Siemens 102 Transmission Electron Microscope at a nominal magnification of ×40000. The precise magnification was calibrated with a diffraction-grating replica. The extensin molecules were clearly seen as elongated structures in the region immediately adjacent to the droplet centre, which is usual by this method. A small amount of smaller material and a limited number of end-to-end aggregates of extensin were also observed.

Analytical ultracentrifugation studies

Ultracentrifugation experiments were performed at 20 °C with a Beckman Optima XL-A analytical ultracentrifuge equipped with scanning absorption optics. Extensin was dissolved in 0.4 M phosphate buffer, pH 7.0. For equilibrium studies, a solution column height of 2 mm in a 12 mm path-length double-sector cell was used. The rotor speed was 7270 g and equilibrium was ascertained by the perfect overlay of two absorbance traces acquired 3 h apart at wavelengths between 276 and 320 nm, depending on solute concentration. Data were corrected for absorption by solvent and low molecular mass co-solute by overspeeding the rotor to pellet out extensin, leaving behind a true optical baseline. For velocity studies, a column height of 11 mm was used. The rotor speed was 65420 g, sufficient to overcome diffusion effects without sedimenting the extensin to the cell base before data (acquired at 30 min intervals) could be recorded.

RESULTS AND DISCUSSION

There have been conflicting reports about the molecular mass of plant extensins. By acid-urea/PAGE and CsCl centrifugation, the molecular masses of extensin-1 and extensin-2 from carrot were estimated [12,18]. Extensin-1 was resolved by acid-urea/PAGE into monomers, dimers and trimers (86, 172 and 258 kDa) and extensin-2 banded as a protein of molecular mass 40.4 kDa. These researchers suggested that the molecular mass of the core protein of extensin-2 is 14.1 kDa, considering that it comprises 65 % carbohydrate. Extensin-1 remained in the stacking gel of an SDS/polyacrylamide gel and extensin-2 banded with an apparent molecular mass of 145 kDa, higher than that determined with acid-urea/PAGE. The buoyant densities of extensin-1 and extensin-2 as determined by CsCl centrifugation were found by them to be 1.455 and 1.463 g/cm³ respectively. It was concluded from these data that extensin-1 and extensin-2 have a similar carbohydrate composition (65 %). Stuart and

Figure 1 MALDI-TOF mass spectrum of native tomato extensin

Varner [10] demonstrated the buoyant density of carrot extensin to be 1.44 g/cm³ and found that it comprised 50% (w/w) carbohydrate. The difference in carbohydrate composition of extensin isolated from aerated carrot root disks reported by the two groups of researchers is likely to have been due to variations in age and aeration time of the tissue. Our finding of 34% carbohydrate [8] reflects differences between carrot and tomato extensin; the latter was isolated from suspension-cultured cells.

Our earlier report on the molecular mass of tomato extensin (in excess of 240 kDa) [8] suggested that extensin was either not monomeric (and presumably contained multiple monomeric units), or that, owing to its rod-like structure, it tumbles to form hydrodynamic spheres with a large effective diameter and thus elutes at a lower elution volume than that expected for its actual molecular size [19]. However, trifluoroacetic acid-deglycosylated extensin was found to have a molecular mass of approx. 240 kDa by gel-filtration chromatography [8]. Deglycosylated extensin loses its extended shape [14,20]. The high molecular mass estimate of our native extensin preparation prompted us to examine its shape. CD spectra of our sample shows a minimum at 200 nm and a maximum at 225 nm, typical of a standard polyproline II conformation with no evidence of α -helix or β -sheet conformations (results not shown). Therefore the rod-like conformation of extensin suggests that the molecule is likely to diffuse rapidly through a gel-filtration matrix, eluting at a lower elution volume and producing an overestimate of its actual molecular mass. It was earlier reported, on the basis of CD spectroscopy, that polyproline, polyhydroxyproline and extensin possess similar extended conformations [20,21].

MALDI-TOF MS was used to generate a shape-independent molecular mass determination for extensin. The spectrum in Figure 1 shows a peak at m/z 72 350, which represents the molecular mass. The second narrower peak at m/z 36 110 has been assigned to M^{2+} , a doubly charged ion. The latter peak is half the pseudo-molecular ion (m/z 72 350) and is notably sharper, which would be expected from the extra charge on the doubly charged ion. The peak at m/z 72 350 is broad with slight shoulders,

which probably reflects carbohydrate microheterogeneity within the extensin sample. However, it is not possible to identify the sugar residues from these shoulder peaks because the peaks remain unresolved. Considering that there is 34% carbohydrate in this tomato extensin preparation [8], the mass of the deglycosylated extensin would be 48 kDa.

Extensin cross-linked *in vitro* (high-molecular-mass multimeric extensin [8]) was also analysed by MALDI-TOF MS. There were no peaks at higher molecular masses (up to 300 kDa). Therefore this form of extensin seems to have a mass in excess of 350 kDa and is beyond the practical limit of determination by this technique. The MALDI-TOF mass spectrum supports the homogeneous nature of our purified extensin preparation. This technique does not suffer from sample suppression, owing to the presence of impurities, to the same extent as other mass spectrometric techniques. Therefore if other components were present we would expect to see evidence of them in the spectrum. This was not the case.

Although gel-filtration will provide a rapid separation between monomeric and oligomeric extensin, transmission electron microscopy will provide precise information of the size, shape and molecular dimensions of extensin. Heckman et al. [19] performed transmission electron microscopy of an extensin preparation derived from sycamore and tomato suspension-cultured cells. Both preparations comprised mainly monomers with a small quantity of oligomers. They found them to be 'flexuous' rods containing apparently random kinks with a contour length of 70–100 nm and a 'persistence length' (maximum linear displacement) of 44–51 nm.

In our investigation, most of the field seems to contain very extended particles that clearly tend to aggregate (Figure 2). The shortest reliably measured particles are approx. 20 nm $\times$ 2–4 nm, which is half the expected length, and wider than predicted from the centrifugation studies. However, a significant number of particles are of length 40–50 nm and width 2–2.5 nm, which agrees well with the hydrodynamic data; we therefore suggest that the shorter particles (which are normally wider) could arise

Figure 2 Electron micrograph of extensin, unidirectionally shadowed at 5 ° with platinum after spraying on mica from 50% glycerol

A droplet centre is at the bottom of the field of view. Images are seen of elongated particles of length 40–50 nm (single arrows) a few of which seem to have 'kinks' in the middle (double arrows). Also seen are a number of shorter (and wider) particles (asterisks) and larger aggregates (large arrows). A number of particles of more globular appearance might represent either a small amount of contaminating species, although there is no evidence for this, or possibly a number of extensin particles degraded during high-pressure spraying and adsorption on mica.

Figure 3 Apparent whole-cell weight-average molecular mass as a function of extensin loading concentration

Three nonlinear least-squares fits are shown: BM fixed at zero, giving $K_d = 0.5 \pm 13.2 \mu\text{M}$ (broken line); BM and K_d floated, giving $BM = 44.7 \pm 10.0 \text{ ml/g}$ and $K_d = 8.5 \pm 2.2 \mu\text{M}$ (solid line); BM and K_d floated and data point for an extensin concentration of $13.7 \mu\text{M}$ excluded, giving $BM = 43.5 \pm 2.0 \text{ ml/g}$ and $K_d = 7.1 \pm 0.4 \mu\text{M}$ (dotted line).

by compaction of the longer particles or folding back on themselves through 180° at the 'kink'. A 'kink' located part of the way along the length of the 40–50 nm particles is observed. This 'kink' is consistent with those 'kinks' observed by Stafstrom and Staehelin [18] and attributed to intramolecular isodityrosine linkages. The larger aggregates might arise during the preparation of the sample for electron microscopy. Unidirectionally shadowed, extended particles seem polydisperse when judged visually or by analysis of the shadow width of electron micrographs. This is so, even for a totally monomeric preparation, because of the variable angle subtended by the shadowing direction with respect to the long axis of the particle [22].

The sedimentation equilibrium solute distribution for extensin was analysed in three ways. First, the IDEAL1 model of the Beckman data analysis software was fitted to the data, yielding the apparent whole-cell weight-average molecular masses [$M_w(\text{app})$] at given loading concentrations. IDEAL1 treats the solute as thermodynamically ideal and monodisperse: the relative poor fit obtained is indicative of non-ideality. It is clear from Figure 3 that, above micromolar concentrations, extensin self-associates to yield weight-average molecular masses significantly in excess of the monomeric 72.3 kDa measured by MALDI-TOF MS (Figure 1). Therefore the Beckman software model ASSOC4 was fitted to the data, permitting the modelling of up to four dissociation constants. The high asymmetry revealed by electron microscopic studies together with the finite non-ideality evident in the initial IDEAL1 analysis restricted ASSOC4 analysis to the lowest concentration systems where the effect of non-ideality was minimal. Excellent fits were obtained, giving dissociation constants, K_d , of 3.22 ± 0.24 , 4.13 ± 0.30 and 5.17 ± 0.87 (S.E.M.) μM for loading concentrations of 4.1, 6.8 and $9.6 \mu\text{M}$ respectively. The concentration dependence of these apparent K_d values is indicative of thermodynamic non-ideality. Thus the final analysis encompassed self-association and non-ideality. For a self-associating system, the weight-average molecular mass (M_w) is related to the monomer mass (M_0) through

the molar loading concentration (C) and K_d in the following manner:

$$M_w = \frac{M_0}{C} [2C + (K_d - \sqrt{K_d^2 + 8CK_d})/4] \quad (1)$$

Non-ideality due to excluded volume effects (expected to be large because of macromolecular asymmetry) and to Donnan effects can (for rod-shaped molecules [23]) be expressed as the sum of these two contributions:

$$BM = L\bar{v}_s/d + 1000vZ^2/4Mm \quad (2)$$

where B is the second virial coefficient, L/d is the axial ratio of the rod, $\bar{v}_s$ is the solvated macromolecular partial specific volume ($\bar{v} + \delta v$), where δ is the hydration (g of solvent/g of extensin) and v is the solvent's specific volume), Z is net macromolecular charge and m is the salt concentration. BM was calculated for extensin monomer and dimer. The axial ratio was estimated from electron microscopy and sedimentation velocity data (see below) to be 20 for the monomer and 16.7 for the dimer. The partial specific volume of extensin was calculated from the known amino acid composition and the estimated carbohydrate content [8] ($\bar{v} = 0.71 \text{ ml/g}$). Similarly, the hydration was estimated (by using data from [24]) to be 0.5 g of solvent/g of protein. Although there are few published data that confirm the elevation of hydration by carbohydrate, our experience with other glycoproteins suggests that δ does increase slightly. Therefore a total hydration of 0.7 g of solvent/g of extensin was assumed. At pH 7.0, extensin has a net charge of 37 and all hydrodynamic experiments were performed in 0.4 M salt; thus $m = 0.4$. Putting these parameters into eqn. (2) gives BM values of 40 and 47 ml/g for monomer and dimer respectively. An average BM of 43.5 ml/g was therefore used to model the experimental data with the combination of eqns. (1) and (3):

$$M_w(\text{app}) = M_w/(1 + BM_w c) \quad (3)$$

where c is the mass loading concentration. Three fits are shown in Figure 3. With BM fixed at zero, the best fit to the data was obtained with $K_d = 20.5 \pm 13.2 \mu\text{M}$, although this fit actually only coincides with experimental data at $4.1 \mu\text{M}$. Floating BM and K_d , two fits were obtained: for all data points ($K_d = 8.5 \pm 2.2 \mu\text{M}$, $BM = 44.7 \pm 10.0 \text{ ml/g}$) and for the exclusion of the $13.7 \mu\text{M}$ point ($K_d = 7.1 \pm 0.4 \mu\text{M}$, $BM = 43.5 \pm 2.0 \text{ ml/g}$). The exclusion of this questionable data point makes little difference to the fit and to the resultant K_d and BM , which is in excellent agreement with the average theoretical BM (43.5 ml/g) estimated above.

Data from sedimentation velocity experiments were analysed in parallel with those from equilibrium experiments. The symmetrical, sigmoidal sedimenting boundaries observed are consistent with either a single solute component or a rapid monomer-dimer equilibrium [25]. The former possibility is ruled out by the lack of the strong negative dependence on concentration expected for an asymmetrical component [26]. To attempt to define the putative monomer-dimer equilibrium we undertook detailed iterative modelling of the data, using the extension of the method of Gilbert and Gilbert [25] described by Emes and Rowe [27]. A slight further extension of this approach that uses the full general equation for concentration-dependence [28] has been incorporated into a program SA-Plot (P. Fellows, University of Leicester).

Before the iterative modelling via SA-Plot, it was necessary to define values for the extrapolated sedimentation coefficients of the monomer and dimer, and hence the k_s values from the assumed solvation values. This was achieved by hydrodynamic bead modelling (with the program HYDRO [29]) and the results

We cannot yet speculate in any detail about the likely spatial

and functional involvement of extensin in the primary cell wall. The model now advanced should, however, form the basis for a more detailed study of these latter topics.

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