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MATRIX ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY OF TOMATO EXTENSIN MONOMERS AND POTATO LECTIN

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Abstract—Extensins are basic hydroxyproline-rich glycoproteins (HRGPs) of the primary cell wall. They exist mainly as an insoluble network, but also as soluble precursors which are readily eluted from intact cultured cells by dilute salt solutions. Although generally regarded as monomeric, these soluble network precursors exhibit anomalous behaviour on gel electrophoresis and gel filtration, which has recently led to an alternative view of soluble extensin as globular-like aggregates of covalently crosslinked monomers. This discrepancy needs to be resolved as it impinges directly on mechanisms of wall assembly. Therefore, using matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) we measured the molecular masses of P1 and P2, two highly glycosylated soluble extensins from tomato. P1 was 89.7 kDa while P2 was 132.1 kDa. MALDI-TOF MS control experiments with the related HRGP, potato lectin, showed that it occurred as a mixture of monomers and dimers. This excludes the likelihood of oligomer disruption and confirms earlier conclusions that soluble extensins exist mostly as monomers. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Hydroxyproline-rich glycoproteins (HRGPs) of the extensin superfamily contribute to the extracellular matrix throughout the plant kingdom [1]. Some are basic, others are acidic. Some are minimally glycosylated ($\leqslant 1.6\%$ sugar, dry wt), others are highly glycosylated. And some form covalently crosslinked networks involved in growth and disease resistance, hence the current interest in extensin crosslinking [2–4].

One approach to crosslinking involves the use of soluble substrates and appropriate enzymes in a cell-free system. The feasibility depends on the 'intact-cell elution' technique [5] which releases a substantial *in muro* pool of extensin network precursors [6]. These consist largely of 90 kDa monomeric, rodlike glycoproteins [7, 8]. Recently, however, Brownleader and Dey [9, 10] have questioned that conclusion; they suggest that extensins eluted from intact cells are not monomeric, but instead predominantly consist of at least 20 monomers *covalently* crosslinked to form large (1500–2000 kDa) oligomers with 'globular-like characteristics'. This discrepancy needs to be resolved as it is relevant to HRGP biosynthesis, macromolecular migration, self-assembly of the wall matrix, and the mechanism by which cells secrete macromolecules of ~90 kDa across a cell wall whose known porosity [11] excludes large globular proteins.

Confusion has arisen because the sizing of asymmetric molecules by standard gel filtration and electrophoretic methods is uncertain. Therefore, we chose a different method based on recent technological advances that permit the accurate 'weighing' of proteins [12, 13], namely matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). We measured the molecular masses of two highly glycosylated extensin monomers from tomato, P1 and P2. These were 89.7 and 132.1 kDa, respectively. We also determined their molecular mass after HF-deglycosylation which, by comparison with the glycosylated mass, yielded an accurate estimate of carbohydrate content. These direct measurements corroborate earlier extensin mass estimates [6, 8, 14, 15] and also confirm the conclusion that intact cell elution does indeed yield largely monomeric extensin. On the other hand, the related HRGP potato lectin gave a monomer–dimer mixture showing that the MALDI-TOF MS conditions were nondisruptive.

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RESULTS AND DISCUSSION

Since their introduction [16] cell suspension cultures have become of great utility in plant biochemistry. Besides the obvious advantages of handling homogeneous pipettable axenic plant tissue, suspension cultures also provide the bonus of an elutable cell surface that yields *soluble* HRGP extensins which are precursors to insoluble cell wall protein [6]. Previous work [6, 7] identified the bulk of these soluble HRGPs as monomeric, (with only a minor fraction (~5%) consisting of small oligomers) as evidenced by gel filtration on Superose-6, also used in the work described here (Fig. 1). However, Brownleader and Dey [9, 10] recently concluded that these HRGPs obtained by elution of intact cells are *not* monomers *per se*. but large (1500–2000 kDa) globular-like oligomers consisting of at least 20 *covalently* crosslinked monomers. Hence our reassessment of this question based on both old and new data.

Firstly, there is the problem of reconciling the size of eluted molecules with the size of the cell wall pores through which they must pass. The oligomers postulated by Brownleader and Dey are much larger than proteins such as BSA (68 kDa) which the cell wall porosity excludes [11, 17]. The cell wall should, therefore, easily exclude globular-like HRGP oligomers from passing through. Yet Smith *et al.* [6] observed a very rapid ionic desorption and elution of HRGPs, which would be most unlikely if they were oligomers. One could postulate a mechanism involving a few large pores, for which there is little or no evidence.

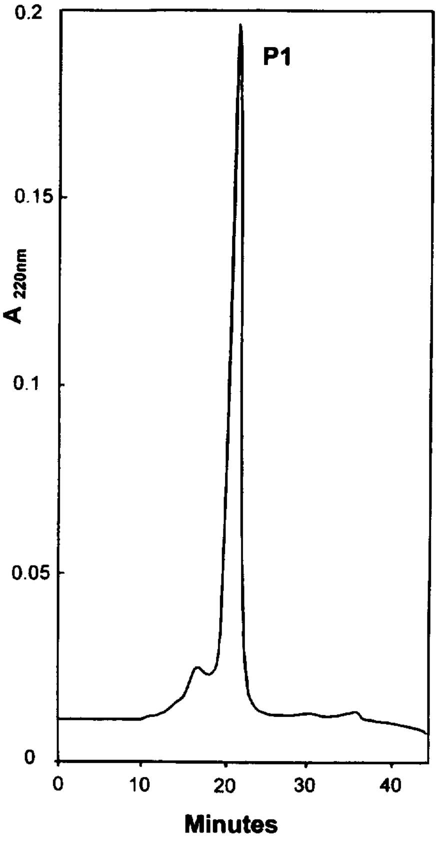

Fig. 1. Superose-6 gel permeation chromatography of tomato extensin P1. After BioRex-70 cation exchange fractionation, P1 was further purified by gel permeation chromatography. We collected the major peak (P1) for molecular mass measurements by MALDI-TOF MS.

Secondly, treatment of the eluted soluble HRGPs with anhydrous HF at 0° yields deglycosylated products of 50–55 kDa (via SDS–PAGE) [6, 18], which fall within the size range of monomeric extensins deduced from cDNA data [19]. On the other hand, covalently crosslinked oligomers produced by *in vitro* crosslinkage [20] yield stable HF-deglycosylated polypeptides that remain oligomeric and do not revert to monomers. This indicates that the crosslink is HF-stable and, therefore, any deglycosylated monomers must originate from native glycosylated monomers rather than oligomers. Incidentally, the HF-stability of the intermolecular crosslink produced *in vitro* probably explains why treatment of isolated cell walls with anhydrous HF leaves an insoluble residue, presumably of crosslinked HRGPs [21], rather than a soluble mixture of monomers; deglycosylated monomers are highly basic, and water soluble, especially at acid pH.

Thirdly, the 69–86 nm contour lengths of various soluble HRGPs measured by transmission electron microscopy after rotary shadowing [7, 8, 15], correspond to a ~250-residue minimum for a polypeptide backbone in the extended (three residues/turn, 9.6 Å pitch) polyproline-II conformation indicated by circular dichroism spectroscopy [15]. Furthermore, simple inspection of the rotary shadowed images shows that they represent rodlike monomers [7, 8, 15].

Taken together, the above approaches provide a general, although not definitive, indication of molecular size. An accurate estimate of molecular weight is uncertain when dealing with the extremes of molecular shape and composition exemplified by HRGPs. Hence, the desirability of an independent and direct measurement afforded by MALDI-TOF, which essentially 'weighs' ions in the gas phase [13], and is not subject to the vagaries of polymeric matrices used for gel electrophoresis or gel filtration. Thus MALDI-TOF MS of two tomato extensins, P1 and P2, and the extensin-like PHRGP from Douglas Fir [22], gave ions whose mass corresponded to the monomer both before and after deglycosylation (Fig. 2, Table 1.). The mass of glycosylated P1 (corresponding to the major Superose-6 peak in Fig. 1) also agreed with the very first estimates of 86–92 kDa obtained by cationic exchange urea gel electrophoresis and sucrose density gradient centrifugation of a similar soluble extensin from carrot root discs [14]. Although these [14] and other workers [8, 15] obtained their material by elution of isolated cell walls rather than intact cells, the close agreement between their data and ours shows that both methods yield similar monomeric material.

It could be argued that the monomers from tomato might represent only a very small proportion of the

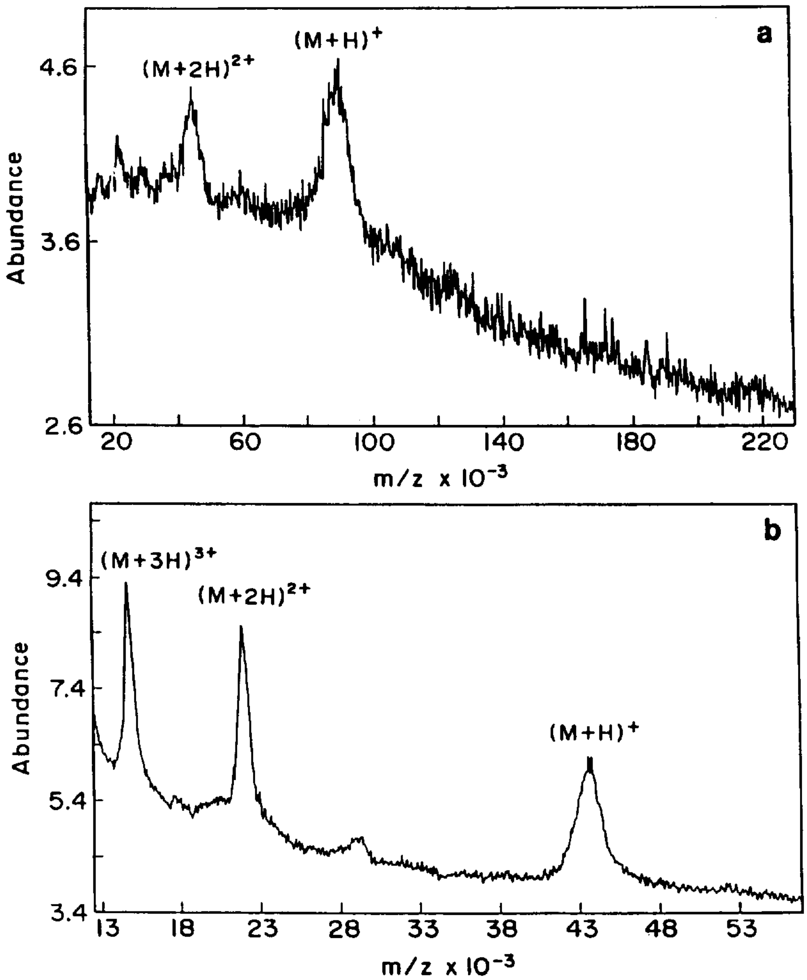

Fig. 2. MALDI-TOF mass spectra of (a) glycosylated and (b) HF-deglycosylated tomato extensin P1. (a) The spectrum of glycosylated P1 contained peaks corresponding to doubly $(M + 2H)^{2+}$ and singly charged $(M + H)^+$ molecular ions centered at m/z 44920, and 89694, respectively. We scanned past m/z 750000 (not shown), but found no evidence of peaks corresponding to multimeric P1. (b) The spectrum of deglycosylated P1 showed three peaks at m/z 14655, 21859 and 43441 corresponding to the triply, doubly and singly charged species, respectively. We calculated from these spectra that P1 carbohydrate accounts for 52% of its dry wt. Judging from the breadth of the peaks of (a) glycosylated P1 and (b) deglycosylated P1, carbohydrate heterogeneity accounts for most of the mass range. We calculated from earlier carbohydrate analyses [28] and from the spectra featured here that P1 contains approximately 287 residues of arabinose and 18 residues of galactose; however, the total number of sugar residues varies by about 43 units (~ 6900 amu).

Superose peak, as the MALDI-TOF range does not extend far enough to include very large (~ 20-mer) oligomeric extensin. Of course this would not explain why monomer should appear at all in a supposedly covalently crosslinked oligomer peak obtained by gel filtration, nor would it explain the absence of small oligomers, in the dimer to pentamer range, which are certainly small enough to be detected by MALDI-TOF MS. Indeed, the closely related HRGP potato lectin [23] exists as a freely reversible equilibrium of monomers and dimers [24, 25], yet we detected both via MALDI-TOF (Fig. 3 and Table 1). Thus, our demonstration of monomeric extensin molecules by MALDI-TOF MS corroborates earlier conclusions and refutes the assertion [9, 10] that salt-eluted extensin precursors are multimers consisting of monomeric extensin covalently crosslinked to other extensin monomers. This confusion arose in part because monomeric extensins behave anomalously compared with normal globular proteins of similar mass, particularly when subjected to standard sizing procedures, notably SDS–PAGE and gel filtration. We explain this anomalous behavior in terms of the highly basic glycosylated rodlike properties of extensin molecules as follows.

Firstly, consider the well-known anomalous behavior of glycoproteins in general on SDS–PAGE [23, 25]. Extensive glycosylation sterically hinders the binding of SDS to the polypeptide backbone [26]. Steric hindrance decreases the net negative charge and hence electrophoretic mobility, compared with that of a similarly sized non-glycosylated protein; this accounts for the poor migration of native extensions on SDS gels. On the other hand, deglycosylated exten-

Table 1. Molecular masses of glycosylated and deglycosylated HRGPs from tomato, carrot, potato and Douglas Fir

HRGP type	Glycosylated mass (Da)	Deglycosylated mass (Da)
Tomato P1	89 694*	43 441* 55 000† [6]
Tomato P2	132 056*	50 194* 53 000† [6]
Brownleader and Dey's tomato extensin	1 500 000–2 000 000‡ [9, 10]	not determined
Carrot extensin	86 000§ [14]	36 000† [15]
Potato lectin: monomer	55 010*	31 431*
Dimer	106 877*	
Douglas fir PHRGP	73 186* [22] 669 000‡ [22]	53 953* [22]

*Determined by MALDI-TOF MS; for glycosylated HRGPs, these weights reflect the center of broad ion peaks (± ~4500 amu on either side of the peak corresponding to about ±21 sugar residues).

†Estimated by SDS–PAGE.

‡Estimated by FPLC gel permeation chromatography.

§Estimated by cationic urea gel electrophoresis and sucrose density gradient centrifugation.

sins migrate well with an apparent mass only slightly greater than their true mass [6]. Unfortunately, Brownleader and Dey [9] could not confirm this last crucial point stating that their 'HF-treated extensin was totally water-insoluble' and they suggested that complete deglycosylation via HF-solvolysis of *O*-glycosidic linkages 'may have generated an intrinsically insoluble molecule'. This is contrary to our experience

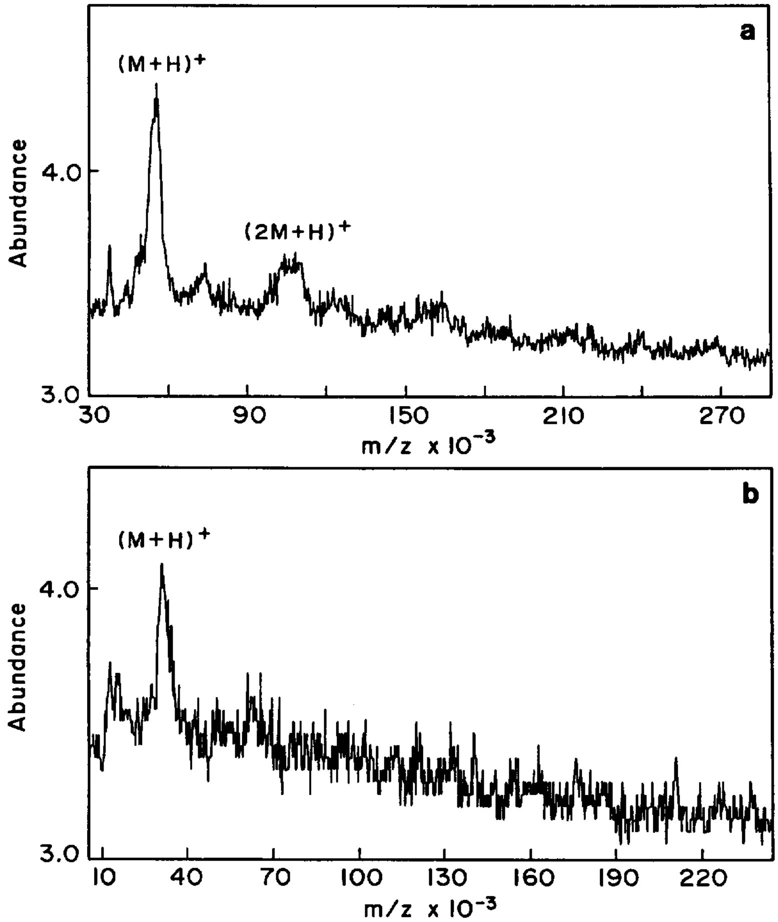

Fig. 3. MALDI-TOF mass spectra of (a) glycosylated and (b) HF-deglycosylated potato lectin. (a) The mass spectrum of glycosylated potato lectin contained two peaks centered at *m/z* 55 010, and 106 877 corresponding to monomeric potato lectin and presumably to dimerized potato lectin, which was characterized earlier [24, 25]. (b) The mass spectrum of HF-deglycosylated potato lectin samples had only one peak corresponding to the deglycosylated monomer.

with extensin monomers both here (Table 1) and in Lamport's laboratory, where more than two dozen extensins and related HRGP samples from 16 species invariably yielded water- or neutral-buffer-soluble products after HF-deglycosylation [6, 7, 18, 20, 22, 29–35, M. Kieliszewski, unpublished data for bean, tobacco, red pine, carrot and soybean; M. Kieliszewski and D. Lamport, unpublished data for Ginkgo]. A single molecular ion obtained after HF-deglycosylation of P1 (Fig. 2 and Table 1) and P2 also indicates that no peptide bond cleavage occurred under these conditions (0) of HF solvolysis, again confirming earlier results [6]. In fact, deglycosylation yields a much sharper MS peak (Fig. 2). This significant decrease in the molecular weight range arose from carbohydrate microheterogeneity. We estimate that the P1 ion peak (Fig. 2(a)) contains ~305 sugar residues, while the extrema contain plus or minus 21 sugar residues.

Secondly, consider the anomalous behaviour of rodlike proteins on gel filtration [36]. The retardation of such highly asymmetric molecules differs significantly from that of similarly sized globular molecules which tumble and can be treated as perfect spheres. Rodlike molecules however, tend to be constrained by entanglement interactions and therefore diffuse by a different mechanism that hypothetically involves endwise 'worm-like movement in a tube', or reptation [37]. Recent observations of single fluorescently labeled molecules confirm reptation theory [38, 39]. Thus, compared with globular proteins of similar mass, anomalous behavior of rodlike molecules on gel filtration may arise from a combination of constraints on tumbling and the requirement for end-on insertion of properly oriented molecules into the pores of the gel matrix [36] as well as flow-polarization; for example, collagens exhibit flow birefringence [40].

These anomalous gel filtration and electrophoretic properties of rodlike extensin monomers are non-trivial, as extensins are designed for insertion into the wall matrix. The rules that govern the migration of highly charged rodlike macromolecules through a polymeric matrix, whether it be agarose, poly(methylmethacrylate), or the primary cell wall, may also explain the movement of extensin monomers into and across the cell wall. We suggest that extensin monomers reptate into the wall matrix, assisted by a transmural electrostatic gradient generated between the positively charged outer surface of the plasma membrane and the negatively charged outer region of the primary cell wall which contains highly de-esterified pectin [41, 42].

Monomer migration is undoubtedly relevant to correct positioning within the wall matrix. However, the structural diversity [1] and spatiotemporal regulation of the extensin HRGP superfamily [43] signify subtler properties and interactions determined by diverse functional domains. For example, those with the Val-Tyr-Lys motif are involved in cross-linking [20]; others such as the 'non-elutable' P3 extensin of tomato [6, 28] contain palindromic peptides (i.e. the side chain sequence is the same when read from either direction); these remarkable motifs may be involved in recognition and self-association [1]. Although this hypothesis remains untested, we note that potato lectin self-associates and has a P3-like domain [23] in addition to a cystine-rich chitin-binding lectin domain related to the ancient disulfide-bridged toxin-agglutinin fold [44]. Thus, the two domains of potato lectin could be structurally and functionally analogous to the animal C-type lectins, which are globular with collagen tails and are involved in first line host defence in mammals [45]. Elucidation of other functional domains of the extensin superfamily remains a challenge for the future.

EXPERIMENTAL

HRGP preparation by intact cell elution and column chromatography. We prepared tomato extensins P1 and P2 by intact cell elution and BioRex-70 cation exchange chromatography followed by Superose-6 (Pharmacia) gel permeation chromatography, as described earlier [7]. We prepared potato lectin by BioRex-70 and reverse phase chromatography, also as described earlier [23].

Deglycosylation of HRGPs with anhydrous hydrogen fluoride (HF). We deglycosylated 1–2 mg of HRGP with HF containing 10% anhydrous methanol for 1 h at 4 , as described earlier [21, 22]. Before application of the samples to the mass spectrometer, we removed traces of HF by reverse-phase chromatography on a Hamilton Polymeric Reverse-Phase (PRP-1; 250 × 4.1 mm) HPLC column equilibrated in Buffer A (0.1% TFA) and eluted at a flow rate of 0.5 mL min^{-1}. The gradient was 0–100% Buffer B/100 min (Buffer B: 0.1% TFA/80% aqueous acetonitrile).

Molecular mass determinations of glycosylated and deglycosylated HRGPs by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). We determined the molecular mass of glycosylated and deglycosylated tomato extensins P1 and P2, and potato lectin on a Hewlett Packard LDI 1700XP mass spectrometer operated at 30 kV accelerating voltage and a pressure of ~6×10^{-7} torr. The mass spectrometer was calibrated with a mixture of equine heart cytochrome C ($M_r = 12\,360$), equine heart myoglobin ($M_r = 16\,950$) and bovine serum albumin ($M_r = 66\,465$). Samples were desorbed/ionized from the probe tip with a nitrogen laser ($\lambda = 337$ nm) having a pulse width of 3 ns and delivering approximately 8 mJ of energy/laser pulse^{-1}. Aqueous solutions of HRGP (10 mg ml^{-1}) were diluted 1 : 8 in aqueous 90% methanol containing 100 mM α-cyano-4-sinnapinic acid. We vacuum crystallized 1 μl of the sample/matrix solution onto the probe, then recorded mass spectra over a m/z range of 1–250 000 using a deflector to attenuate the abundance of ions below 10 000 m/z.

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