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Spinach extensin exhibits characteristics of an adhesive polymer

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SUMMARY

We have investigated the possibility that extensins (basic, hydroxyproline-rich glycoproteins of the primary cell wall) contribute to cellular adhesion. Soluble [^{14}C]extensin, obtained by salt-elution from the walls of living spinach (*Spinacia oleracea* L.) cells, was found to adhere strongly to all surfaces tested, including glass, polypropylene and polycarbonate. The adhesion was not prevented by salts or dilute acids, by urea (a neutral chaotropic agent), or by ascorbate (a reducing agent), which indicates that it was not due solely to ionic bonding, hydrogen-bonding or oxidative coupling. The adhesion was prevented by guanidinium thiocyanate, an ionic chaotropic agent, suggesting that ionic bonding and hydrogen-bonding co-operated in the adhesion. Sodium dodecyl sulphate and two polyanions (poly-L-glutamate and poly-D-galacturonate) each interfered in the adhesion; this is interpreted as being due to their ability to neutralize the positive charge of extensin. Three polycations (poly-L-lysine, poly-L-arginine and poly-L-histidine) each interfered in the adhesion, probably by competing with extensin for binding sites on a glass surface. The data are compatible with the view that extensins are adhesive polymers that could play a role in the binding of cells to each other and to apparently inert surfaces.

INTRODUCTION

Cellular adhesion is important in plant morphogenesis. *Sister* cells, after a cell division, usually remain in firm contact through a middle lamella; this fixes their relative positions, enabling tissue construction. Cell–cell bonds are particularly strong in vascular and epidermal tissues. Where cell separation does occur, e.g. during the formation of air spaces and schizogenous glands and in abscission, the process is highly co-ordinated in space and time (Martens 1937; Sifton 1957; Wooding & Northcote 1965; Kollöffel & Linssen 1984) and may involve dissolution of both the middle lamella and the primary wall.

Non-sister cells may adhere *de novo*: for instance, at the stock–scion interface in grafting, one of the first events is a non-specific cell–cell adhesion (Jeffree & Yeoman 1983). The parasitic plant *Cuscuta* spp. (dodder) can adhere tightly to an inert object e.g. a bamboo cane or a match-stick as well as to its host (Schofield 1987); the adventitious roots of

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Hedera spp. (ivy) adhere to stone walls, and cultured plant cells will often adhere to their glass culture vessels.

Pectins may contribute to cell adhesion by forming pectin–pectin cross-links (Jarvis 1984; Fry 1989; Brown & Fry 1993), but pectins are only partly responsible, as shown by the fact that pectinase is a much less effective macerating agent alone than when supplemented with an unidentified factor (Ishii 1977, 1984). Few tissues are macerated by cold chelating agents, although these can break almost all Ca^{2+}-bridges (Jarvis 1982). Treatment of cultured cells with cold alkali rarely causes maceration, despite solubilizing many polymers. Histological macerating agents include 10% chromic acid/10% acetic acid (heating for $\geqslant 24$ h), H_2O_2/acetic acid (1:1) (boiling for 1 h) and 5% CrO_3 (at 70°C for 2–15 min) (Johansen 1940; Street 1977); these treatments degrade many polymers.

The present paper explores the possibility that extensins have adhesive properties. Extensins are positively charged, extended glycoproteins with a polypeptide backbone rich in hydroxyproline, lysine and tyrosine (Smith *et al.* 1986; Chen & Varner 1985; Caelles *et al.* 1992). Many of the hydroxyproline residues are substituted with a tetra-L-arabinoside (Akiyama *et al.* 1980), and some of the serine residues are α-D-galactosylated (Lamport *et al.* 1973).

Several observations are compatible with a role for extensins in adhesion. Extensins are produced copiously in carrot root slices (Stuart & Varner 1980) and in cell cultures (Lamport & Northcote 1960), both of which may be striving to (re-)form cell–cell bonds. Extensin synthesis is induced upon infection (Esquerré-Tugayé *et al.* 1979; Rumeau *et al.* 1990) and extensins can agglutinate bacteria (Leach *et al.* 1982). In healthy plant organs, extensins are often concentrated in the vascular bundles and epidermis (Ye & Varner 1991; Hood *et al.* 1991), tissues with strong inter-cellular adhesion.

One way in which extensins could contribute to adhesion would be by oxidative coupling to yield isodityrosine cross-links (Fry 1982). Similarly, Vreeland *et al.* (1992) have suggested that the peroxidase-catalysed oxidation of phenolics is responsible for the adhesion of *Fucus* sp. zygotes to rocks. However, we show here that non-oxidized extensins can form strong non-covalent bonds with many inert surfaces.

MATERIALS AND METHODS

Preparation of [^{14}C]extensin

Cell suspension cultures of spinach (*Spinacia oleracea*) were grown, and labelled with [^{14}C]tyrosine, as described by Miller & Fry (1992). To 30 ml of a day-6 cell suspension (settled cell volume 20%) was added 50 μCi of L-[U-^{14}C]tyrosine and 5 ml of 280 mM ascorbate (Na^+, pH 4·0). The suspension was incubated at 25°C for 3·5 h then filtered on nylon gauze and the cells were transferred into 30 ml of 0·1 M $CaCl_2$/25 mM ascorbate (Na^+, pH 4·0), incubated for a further 30 min and filtered again. The filtrate was dialysed against 2 litres of 12 mM 2-mercaptoethanol at 0°C and the retentate stored at −20°C.

Gel electrophoresis

Acid–urea polyacrylamide gel electrophoresis (Stafstrom & Staehelin 1986a) of portions of the [^{14}C]extensin was followed by staining with $AgNO_3$. SDS polyacrylamide gel electrophoresis (Laemmli 1970) was followed by staining with Coomassie Brilliant Blue.

Amino acid analysis

An aliquot of the [^{14}C]extensin was dried and hydrolysed in 6 M HCl (containing 10 mM phenol) at 110°C for 24 h. The products were dried and re-dissolved in 2 M NH_3; aliquots were chromatographed on Whatman No. 1 paper in butan-1-ol/acetic acid/water 12:3:5). One track was stained with isatin/ninhydrin reagent (Kolor & Roberts 1957; modified by Fry 1988) and the intensities of the spots were compared semi-quantitatively with the spots produced by a dilution series of equimolar Lys, Hyp, Pro, Tyr, Val and Ile chromatographed on the same sheet. The second track was assayed for ^{14}C.

Binding of extensin to glasses and plastics

Aliquots (1·0 or 0·1 ml) of a solution containing 106 Bq ml^{-1} (2·4 μg ml^{-1}) [^{14}C]extensin, 80 mM MES (Na^+, pH 6·1) and 0·05% chlorbutol were added to glass or plastic vessels containing 3 volumes of 0·05% chlorbutol with or without SDS, polylysine or other additives. The vessels were then closed and incubated, usually for 18 h at 25°C. To some of the vessels, 'inert' materials were added so as to increase the area available for binding. After incubation, the solution was vortexed and duplicate aliquots were assayed for ^{14}C. Each experiment was carried out at least twice, with similar results; data of one repeat are shown.

Vessels used were: 25-ml 'Pyrex' glass conical flasks (J. Bibby Science Products Ltd., U.K.); 12 × 70-mm soda-glass test tubes (Samco, New York); 60-ml polycarbonate beakers (Sterilin); 1·25-ml polypropylene screw-cap tubes (Sarstedt, Leicester); 1·5-ml polypropylene Eppendorf tubes (Treff Lab, Degersheim, Switzerland); and polyethylene scintillation vials (Zinsser Analytic GmbH, Frankfurt).

Scintillation counting

Strips of chromatography paper were assayed for ^{14}C by scintillation counting (efficiency ~60%) in toluene containing 0·5% PPO and 0·05% POPOP. Aqueous solutions containing ^{14}C were diluted to 2 ml with water, mixed with 20 ml of 0·33% PPO/0·033% POPOP in toluene/Triton X-100 (2:1, v/v) and assayed by liquid scintillation counting at an efficiency of ~75%. Background (~20 cpm) was subtracted. Gel electrophoretograms were scanned with a 'RITA' radiochromatogram scanner (Raytest Instruments, Sheffield).

RESULTS

Partial characterization of spinach [^{14}C]extensin

The ^{14}C-material from the spinach cells was >85% [^{14}C]extensin as judged by its ability to remain bound to SP-Sephadex C-50 at pH 9·0 but to be eluted at pH 9·5 (data not shown; cf. Biggs & Fry 1990). Acid–urea gel electrophoresis showed a single major peak of ^{14}C co-migrating with the major stainable band of extensin (Fig. 1a). SDS gel electrophoresis showed a single major magenta-stained band (data not shown) contrasting with the blue colour given by most proteins.

Semi-quantitative analysis of the major amino-acid residues of the extensin used in the present work (Table 1) broadly agreed with the composition determined more precisely for a similar preparation of spinach extensin (Biggs & Fry 1990).

About 80% of the ^{14}C was in tyrosine after acid hydrolysis (Fig. 1b). The specific activity of the tyrosine obtained was ~68 kBq μmol^{-1}. Thus, assuming that the

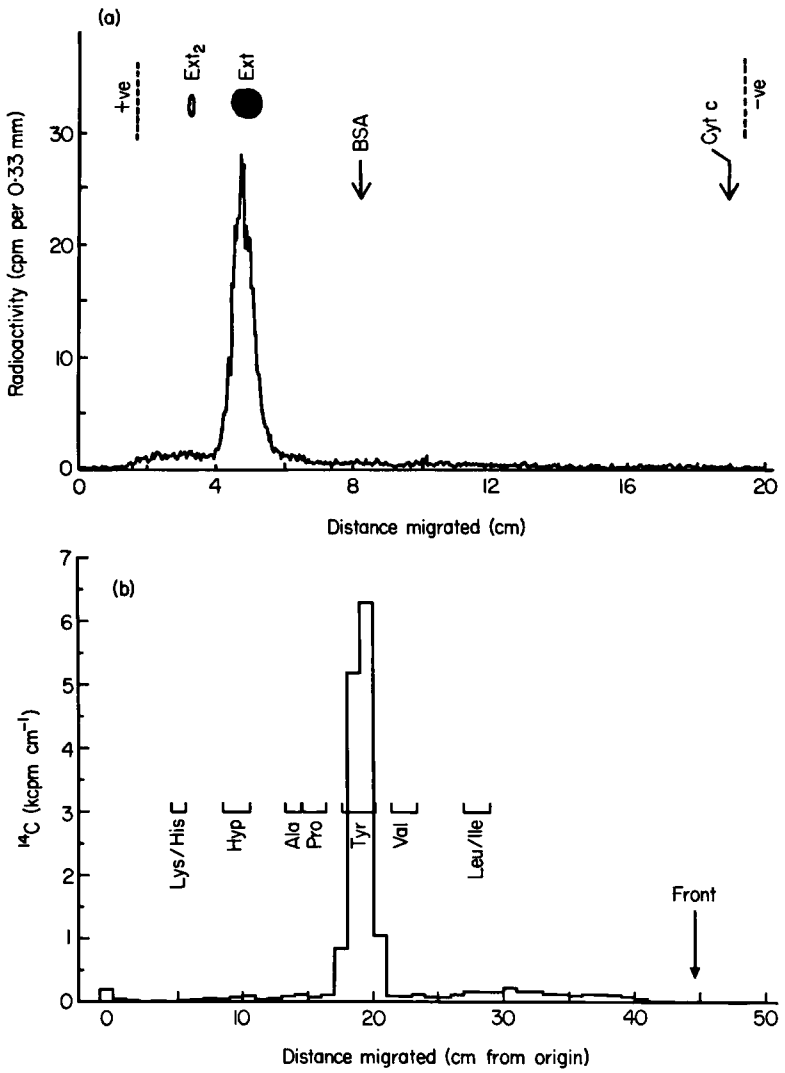

Fig. 1. Partial characterization of spinach [^{14}C]extensin. (a) ^{14}C-profile of an acid–urea gel electrophoretogram of [^{14}C]extensin. The drawing shows the silver-staining pattern of the radioactive sample, which contained a major band of extensin (●) and a faint band of a putative extensin dimer (○). The left and right dashed lines represent the top and bottom of the separating gel, respectively; the first 1·7 cm was stacking gel; the cathode was at the bottom. Arrows (↓) show the position of non-radioactive markers (bovine serum albumin and cytochrome *c*). (b) ^{14}C-Profile of an acid-hydrolysate of [^{14}C]extensin. Brackets (⌴) indicate the R_F values of some major amino acids present in the hydrolysate.

[^{14}C]extensin was roughly 10·5% (w/w) tyrosine residues (Stuart & Varner 1980; Biggs & Fry 1990), the glycoprotein would have a specific activity of $\sim$44 Bq μg^{-1}.

Binding of extensin to glasses and plastics

A substantial proportion of [^{14}C]extensin adhered to all vessels tested (Table 2). The proportion bound was 50% (soda-glass tubes) to 85% (polyethylene vials). When the

Table 1. Approximate ratios of the major amino-acid residues of the [^{14}C]extensin used in the present work, compared to those reported for spinach extensin*

Amino-acid residue	Composition (mol%)	
	Lit.*	Found‡
Hyp	31·80	29
Tyr	14·51	13
Val	11·16	13
Lys+His	14·07	12
Ser	7·22	7
Pro	6·78	5

*Biggs & Fry (1990); quantitative analyses by HPLC.
‡Present work; semi-quantitative analyses by paper chromatography.

Table 2. Binding of extensin to glass and plastic surfaces. Each vessel contained 106 Bq (2·4 μg) of [^{14}C]extensin in 4 ml buffer ± 1·5% SDS ± added plastic or glass material. The ^{14}C remaining in the buffer was assayed after 18 h

Vessel	Added material	[^{14}C]extensin remaining unbound to surfaces after 16 h (Bq)	
		−SDS	+SDS
Pyrex glass flask	—	26·8	108·6
Polycarbonate beaker	—	33·5	104·6
Sarstedt tube	—	29·4	103·0
Eppendorf tube	—	28·8	98·4
Polyethylene vial (5 ml)	—	21·8	100·9
Polyethylene vial (22 ml)	—	15·9	84·0
Soda-glass tube	—	52·5	98·0
Soda-glass tube	Soda-glass*	36·1	101·3
Soda-glass tube	Polypropylene‡	37·2	107·7
Soda-glass tube	PTFE§	40·8	104·2
Soda-glass tube	Polypropylene‖	30·4	99·1

*Splinters from crushed soda glass.
‡Finely sliced 'yellow pipette-tips'.
§PTFE tubing (Aldrich Chemical Co.).
‖Porous plastic frit from a disposable 'Polyprep' column (BioRad).

[^{14}C]extensin in the soda-glass tubes was in contact with additional surfaces (polypropylene, PTFE or soda-glass), appreciably more ^{14}C was lost from solution. The binding in each case was largely prevented by SDS (Table 2).

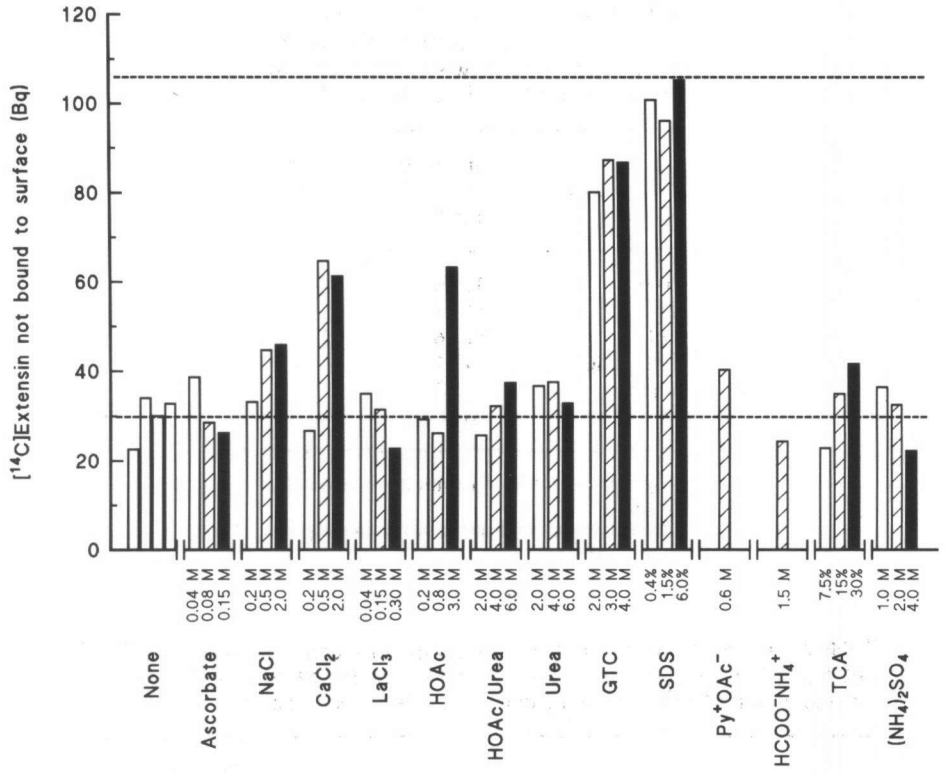

Fig. 2. Effect of added solutes (abscissa) on the binding of [^{14}C]extensin to a polycarbonate surface. Each 'Sterilin' polycarbonate beaker contained 106 Bq (2·4 μg) of [^{14}C]extensin in 4 ml buffer either without additives ('None'; 4 replicates) or in the presence of one of the additives indicated. Each additive was introduced (at progressively increasing concentrations, indicated by □, ▨ and ■) 1 h after the [^{14}C]extensin. The ^{14}C remaining in the buffer was assayed after 18 h. GTC = guanidinium thiocyanate; HOAc = acetic acid; HOAc/Urea = 0·8 M acetic acid containing the urea concentration stated; Py^+OAc^- = pyridinium acetate; SDS = sodium dodecyl sulphate; TCA = trichloroacetic acid.

Effect of low-M_r solutes on binding

To investigate the nature of the [^{14}C]extensin–polycarbonate binding, we sought various solutes that could prevent the binding (Fig. 2). An ionic chaotropic agent, guanidinium thiocyanate (2–4 M), was almost as effective as SDS, but a non-ionic chaotropic agent, urea (2–6 M), had little effect either in the presence or in the absence of 0·75 M acetic acid. Some salts had an inhibitory effect on binding, but in general, salts were not effective. Acetic acid was moderately effective at 3 M, but not at lower concentrations. Trichloroacetic acid, which precipitates many proteins [but not extensin (Heckman *et al.* 1988)], had little effect. Ascorbate was ineffective (Fig. 2), as were 2-mercaptoethanol and dithiothreitol (data not shown), which indicates that the binding was not due to oxidation of the extensin.

Effect of competing polymers on binding

Experiments were conducted to discover what non-radioactive polymers would compete with [^{14}C]extensin for binding to soda-glass surfaces (Fig. 3). Neutral and weakly acidic polysaccharides (dextran, soluble starch and pectin) had little effect. A strongly acidic polysaccharide (poly-D-galacturonic acid) was effective at 0·75% but not at 0·075%. A

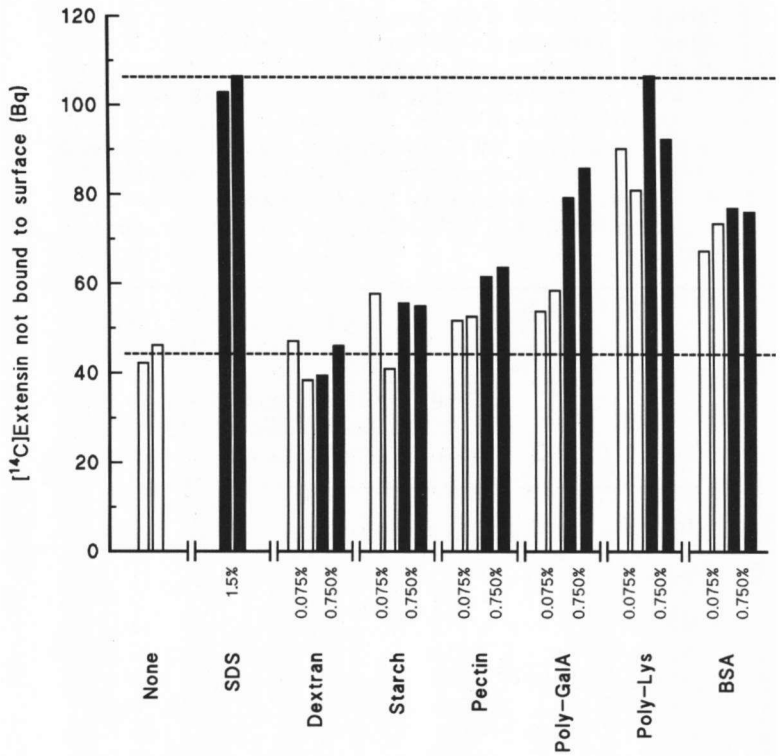

Fig. 3. Effect of added non-radioactive polymers on the binding of [^{14}C]extensin to soda-glass. The non-radioactive polymer indicated on the abscissa (3 ml, pre-adjusted to pH 6·1 with HCl or NaOH, and containing 0·05% chlorbutol) was added to the soda-glass tube 0·5 h before the [^{14}C]extensin [106 Bq (2·4 μg) in 1 ml of buffer, pH 6·1]. The ^{14}C remaining in the buffer solution was assayed after 18 h. Dextran was $M_r \sim 9400$; Poly-GalA = sodium poly-D-galacturonate, from Citrus (Sigma Chemical Co.); Poly-Lys = poly-L-lysine hydrobromide, DP 18 (Sigma Chemical Co.); BSA = bovine serum albumin.

protein (bovine serum albumin; p$I \simeq 5$) was moderately effective at 0·1–1·0%. The most effective competing polymer was a polycation, poly-L-lysine, which almost completely prevented binding.

Three polycations (poly-L-lysine, poly-L-arginine and poly-L-histidine) were all effective, at 0·075–0·75%, regardless of M_r (Table 3). An anionic poly-amino acid (poly-L-glutamic acid) was, like poly-D-galacturonic acid, less effective (Table 3). Another neutral polymer, polyethylene glycol ($M_r \sim 20\,000$), was also weakly effective (data not shown).

Polycations could suppress [^{14}C]extensin binding *either* (1) by modifying the conformation of [^{14}C]extensin to prevent its interaction with glass, *or* (2) by binding to sites on the glass to exclude the [^{14}C]extensin. In (1), the polycation would need to be present in solution simultaneously with the [^{14}C]extensin; this is unlikely to be the case in (2). To test this, soda-glass tubes were treated with non-radioactive polymers and then either rinsed with water or not rinsed. The binding of [^{14}C]extensin to the glass was then monitored (Table 3). The inhibitory effect of the polycations on [^{14}C]extensin-binding persisted after rinsing, which indicates that a lasting blockage of surface sites had occurred.

The effects of SDS and poly-L-glutamic acid (but not poly-D-galacturonic acid) were abolished by rinsing (Table 3). These agents may thus have acted by binding to the extensin molecules to form a complex that was unable to bind glass.

Table 3. Lasting effect of poly-ions on the binding of [^{14}C]extensin to soda-glass. The non-radioactive polymer indicated (0·3 ml of solution, pre-adjusted to pH 6·1 with HCl or NaOH, and containing 0·5% chlorbutol) was added to the soda-glass tubes, which were then closed and shaken for 5 h at 25°C. Some of the tubes were then emptied, rinsed with 5 ml of distilled water and emptied again, and 0·3 ml of 0·05% chlorbutol was added. [^{14}C]Extensin [10·6 Bq (0·24 μg) in 0·1 ml of buffer, pH 6·1] was then added to each tube and incubated at 25°C. The ^{14}C remaining in the buffer solution was assayed after 16 h

			[^{14}C]Extensin *not* bound to soda-glass surface (Bq)	
Additive	M_r	Final conc. (%)	Additive removed before addition of [^{14}C]extensin	Additive not removed
None	—	—	—	5·0
SDS		0·750	5·8	10·4
Poly-Lys*	21 500	0·075	9·7	11·3
		0·750	10·5	9·7
Poly-Lys*	390 000	0·075	10·1	10·3
		0·750	9·4	10·2
Poly-Arg‡	12 000	0·075	7·8	10·4
		0·750	10·3	9·4
Poly-Arg‡	115 000	0·075	9·9	10·3
		0·750	10·5	10·7
Poly-His‡	15 300	0·075	9·7	10·1
		0·750	10·5	9·8
Poly-Glu§	36 200	0·075	4·5	7·9
		0·750	4·5	7·1
Poly-GalA§		0·075	6·6	5·2
		0·750	8·5	8·7

*Weighed as the bromide salt.
‡Weighed as the chloride salt.
§Weighed as the sodium salt.

The [^{14}C]extensin-binding sites on soda-glass could be lastingly blocked by poly-L-lysine solutions as dilute as 0·023%; 0·008% was partially effective (Fig. 4). Poly-L-lysine also blocked the binding of [^{14}C]extensin to polypropylene surfaces (Eppendorf vials), but this effect was only lasting when relatively high concentrations ($\geq$0·225%) of polylysine were used (Fig. 4).

DISCUSSION

Extensin had a remarkable ability to bind to 'inert' surfaces. The binding was not due to oxidative reactions since it could not be prevented by reducing agents. The binding was probably not simple hydrogen-bonding because it was resistant to concentrated aqueous urea. Since extensin has a very high p*I* (Stuart & Varner 1980), it might bind ionically to acidic groups present on the surfaces of glasses and plastics. However, most salts [e.g.

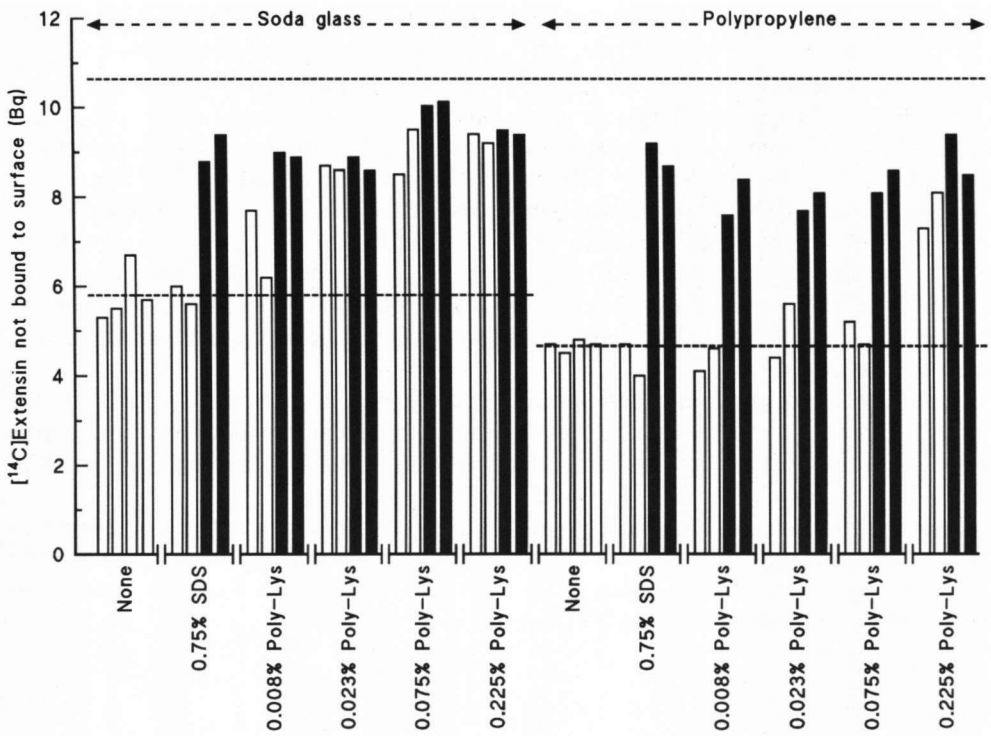

Fig. 4. Concentration dependence, and permanence, of the effect of additives on the binding of [^{14}C]extensin to soda-glass and polypropylene. A solution of poly-L-lysine · HBr (M_r ~21 500) or SDS (0·3 ml, pre-adjusted to pH 6·1 with HCl or NaOH, and containing 0·05% chlorbutol) was added to soda-glass tubes (left half of diagram) or polypropylene Eppendorf tubes (right half), which were then closed and shaken for 5 h at 25°C. Some of the tubes (□) were then emptied, rinsed with distilled water and emptied again, and 0·3 ml of 0·05% chlorbutol was added back. In the other tubes (■), the SDS or Poly-Lys solution was left undisturbed. [^{14}C]Extensin [10·6 Bq (0·24 μg) in 0·1 ml of buffer, pH 6·1] was then added to each tube and incubated at 25°C. The ^{14}C remaining in the buffer solution was assayed after 18 h.

those that efficiently solubilize ionically-bound extensin from the cell wall (Smith *et al.* 1984)] and acids did not prevent binding. Since the binding was prevented by an ionic chaotropic agent (guanidinium thiocyanate) but not by a non-ionic one (urea) nor by non-chaotropic salts (e.g. NaCl), it seems possible that ionic and hydrogen bonds contributed jointly to the binding of [^{14}C]extensin. Several other lines of evidence suggest a role for ionic bonding:

(a) The binding was prevented by the simultaneous presence of SDS, which gives polypeptides a negative charge. (Spinach extensin migrated towards the anode on SDS gel electrophoresis, indicating that SDS overcame even the strong positive charge of native spinach extensin.)

(b) Binding was partially prevented by the simultaneous presence of poly-L-glutamate or poly-D-galacturonate, which may bind ionically to [^{14}C]extensin, forming a complex with little or no net charge.

(c) *N*-Succinylation of [^{14}C]extensin, which converts it to a polyanion (Glazer *et al.* 1975), prevented binding to glass and plastics (data not shown). (This observation also suggests that the carbohydrate components of extensin were not sufficient for adhesion.)

(d) The binding sites of glass and plastic surfaces were lastingly blocked by treatment with other polycations, e.g. poly-L-lysine, poly-L-arginine and poly-L-histidine. Neutral polymers and polyanions had little or no lasting effect on glass or plastic surfaces.

It is clear that the preliminary data presented here do not establish the precise nature of the interaction between extensin and 'inert' surfaces. Nevertheless, it is interesting to consider the possible significance of this interaction for plant cell adhesion. Extensins are rod-like molecules about 80-nm long (Stafstrom & Staehelin 1986b). At least part of the extensin molecule becomes covalently bound within the primary cell wall, probably via isodityrosine cross-links (Fry 1982; Biggs & Fry 1990). It seems plausible that portions of some of the wall-anchored extensin molecules protrude out of the cell wall. Protruding portions may, as shown here, adhere to foreign surfaces, including those as apparently inert as glass and plastics.

Possible roles for extensin adhesion include the attachment of the middle lamella to the primary cell wall, the attachment of the cuticle to the outer epidermal wall, the immobilization of pathogens, the early bonding of tissues during grafting and the attachment of specialized adhesive organs to stone, wood and other surfaces.

Since its discovery (Lamport & Northcote 1960), extensin has been considered as an architectural element *within* the structure of primary cell walls, influencing their extensibility and digestibility. The present findings raise the possibility that it may also have roles in establishing higher-order features of plant anatomy.

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