Galactosylserine in Extensin

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Cell walls obtained from tomato suspension cultures were treated at pH 1 for 1 h at 100°C to remove arabinose oligosaccharide substituents from the hydroxyproline residues of extensin. Tryptic attack of these acid-stripped walls yielded glycopeptides containing galactose. When one of these glycopeptides (designated S₂A₆; sequence NH₂-Ser-Hyp-Hyp-Hyp-Hyp-Ser-Hyp-Lys-CO₂H) was treated with (a) NaOH-NaBH₄ or (b) NaOH-Na₂SO₃ some of the serine was converted into (a) alanine or (b) cysteic acid, and the peptide lost galactose. Maleylation or 3-carboxypropionylation of *N*-terminal serine was necessary for conversion of this residue and for complete loss of galactose. These results indicate that a single galactose residue is attached *O*-glycosidically to each of the two serine residues. Hydrazinolysis of peptide S₂A₆ or of isolated cell walls also led to destruction of serine. In control experiments non-glycosylated serine was not destroyed during hydrazinolysis. Thus the galactosylserine linkage is sensitive to N₂H₄.

Although most workers generally accept that the hydroxyproline-rich protein 'extensin' occurs as an integral component of the primary cell wall of plants, the role of this component is subject to speculation. We favour the hypothesis that extensin is involved in control of cell-wall extension through its involvement in a protein-glycan network (Lamport, 1962, 1971; Lamport & Miller, 1971). Evidence for such a network is gradually being accumulated (Lamport, 1965, 1970; Keegstra *et al.*, 1972), although Heath & Northcote (1971) have questioned the possibility of extensin acting as a cross-link between polysaccharides.

In earlier work (Lamport, 1967; Lamport & Miller, 1971) with cell walls prepared from higher plants we isolated a series of hydroxyproline arabinosides (Hyp-Ara_{*n*}, where *n* varied from 1 to 4) in which the mono-, di-, tri- and tetra-arabinosyl residues were attached glycosidically to the hydroxyproline hydroxyl group. The hydroxyproline arabinosides were obtained by Ba(OH)₂ hydrolysis either of cell walls or of glycopeptides previously obtained by digestion of the walls with a crude cellulase-protease mixture (Lamport, 1969). The glycopeptides also contained galactosyl residues whose position was not defined; mild alkaline conditions did not lead to the elimination of these galactosyl residues, and vigorous Ba(OH)₂ hydrolysis yielded neither hydroxyproline galactosides nor hydroxyproline hetero-oligosaccharides of arabinose and galactose. The hypothesis (Lamport, 1970) that the galactosyl residues were attached via an anomalously alkali-labile linkage to the hydroxyproline arabinosides was made untenable by our recent isolation (Lamport, 1972) of hydroxy-

proline-rich galactoglycopeptides which contained no arabinose. Evidence presented here shows that one of these hydroxyproline-rich glycopeptides, with the sequence Ser-Hyp-Hyp-Hyp-Hyp-Ser-Hyp-Lys, contains a single galactose residue attached *O*-glycosidically to each of the two serine residues.

Materials and Methods

Cell suspension cultures of tomato (*Lycopersicon esculentum* Mill, 'Bonny Best') were grown as described previously (Lamport, 1964).

Cells were blended in the presence of 60 mM-ascorbic acid or 4 mM-sodium metabisulphite, then sonically disrupted, and the cell walls collected by filtration and washed with 1 M-NaCl and water as described by Lamport (1969).

Washed cell walls (approx. 5 mg/ml dry wt.) were adjusted to pH 1 with HCl, and refluxed for 1 h at 100°C to cleave the arabinosylhydroxyproline linkage. The hot suspension of cell walls was then filtered through Whatman no. 1 filter paper, washed with cold water and then rapidly resuspended in water and adjusted to pH 8 with 1 M-NaOH. Trypsin (5 mg) was immediately added to the stirred suspension (approx. 1 g of stripped cell walls in 160 ml of water containing 15 mM-CaCl₂) and the pH was monitored for 1 h at room temperature (23°C) and adjusted to pH 8 if necessary. Tryptic digestion was allowed to continue for up to 18 h. The soluble material was then fractionated by gel filtration on Sephadex G-25 and the sulphonated polystyrene Aminex (AG 50W; X2; 200-325 mesh) as described previously (Lamport, 1971). The peptide S₂A₆ was

further purified by chromatography on a column (0.9 cm $\times$ 27 cm) of Aminex A-5 (Bio-Rad, Richmond, Calif., U.S.A.) in pyridine-acetate buffers (Fig. 1).

Analytical methods

Automated amino acid, hydroxyproline and hydroxyproline glycoside analyses have been described (Lamport, 1969; Lamport & Miller, 1971). Paper electrophoresis was performed by the methods of Lamport (1965), with 2.5% (v/v) formic acid–8.7% (v/v) acetic acid (pH 1.9) and 10% (v/v) pyridine–0.4% (v/v) acetic acid (pH 6.5) with light petroleum (b.p. 170°C) as coolant. Net peptide charge at pH 6.5 was estimated by a graphical method (Offord, 1966).

Identification and assay of D-galactose

The peptides were hydrolysed in 2M-trifluoroacetic acid for 1 h at 121°C and evaporated to dryness in a

stream of N₂ at room temperature. (These conditions gave approximately 10% degradation of free galactose in control experiments.) Galactose was identified in the hydrolysate by: (1) g.l.c. of the alditol acetate after NaBH₄ reduction and acetylation (Albersheim *et al.*, 1967); (2) paper chromatography in ethyl acetate–pyridine–water (8:2:1, by vol.) followed by development with alkaline AgNO₃ (Trevelyan *et al.*, 1950) or *p*-anisidine (Bacon & Cheshire, 1971).

Galactose was also assayed after hydrolysis of peptides in 2M-trifluoroacetic acid, by using Galactostat (Worthington Biochemical Corp., Freehold, N.J., U.S.A.), and in untreated peptides by the cysteine–H₂SO₄ method (Dische, 1962).

Reaction of amino groups

Cyanoethylation with acrylonitrile was used for subtractive *N*-terminus identification (Fletcher, 1966). Amino groups were also maleylated (Butler *et al.*, 1969) and 3-carboxypropionylated (Klotz, 1967).

Fig. 1. Partial acid hydrolysis of Ser-Hyp₄-Ser-Hyp-Lys(Gal₂)

Glycopeptide S₂A₆ (2.3 μ mol) was heated at 100°C for 10 min with 200 μ l of 6M-HCl and then evaporated to dryness in a stream of N₂. The hydrolysate was taken up in 1 ml of pyridine-acetate buffer, pH 2.7, fractionated on a column (0.9 cm $\times$ 27 cm) of Aminex A-5 and eluted with a gradient generated in a gradient maker. Chambers 1–4 each contained 70 ml of 0.07M-pyridine-acetate buffer, pH 2.7, 5 and 6 each contained 70 ml of 0.2M-pyridine-acetate, pH 3.1, and 7 and 8 each contained 70 ml of 2.0M-pyridine-acetate buffer, pH 5.0. All molarities refer to the pyridine. The column was kept at 55°C and eluted at 64 ml/h. Fractions were collected every 3 min. Samples (100 μ l) were assayed by direct reaction with ninhydrin on a modified amino acid analyser. Additional 100 μ l samples were also analysed on the hydroxyproline analyser previously described (Lamport & Miller, 1971). Peaks were pooled and samples taken for amino acid analysis and subtractive *N*-terminus determination by reaction with acrylonitrile (Table 1). ----, Hydroxyproline; —, α -amino acid.

Reaction of peptide and cell walls with hydrazine and NaOH

Peptide or cell walls were placed in a 1 ml microvial (Precision Sampling Corp.) with 200 μ l of N_2H_4 and heated for 10h at 105°C. N_2H_4 was removed in a stream of N_2 and the residue dried overnight *in vacuo* over P_2O_5 .

Peptide or cell walls were treated with a sufficiently small amount of NaOH such that the salt produced by neutralization with HCl did not interfere with the amino acid analyses after hydrolysis in 6M-HCl.

Results and Discussion*Peptide purification and sequence of peptide S_2A_6*

The peptide selected for detailed scrutiny contains two galactose residues and is designated S_2A_6 from its elution position on Sephadex G-25 and Aminex cation-exchange resin. Peptide S_2A_6 was not degraded further by Pronase, and the sequence briefly reported by Lamport *et al.* (1971) was based on partial acid hydrolysis (6M-HCl, 10min at 100°C) and isolation of only two major peptide fragments (Fig. 1), namely Ser-Hyp₄ and Ser-Hyp-Lys (Table 1). Fletcher's

(1966) subtractive method showed that both peptide fragments contained *N*-terminal serine. Ser-Hyp-Lys was degraded further by partial acid hydrolysis (6M-HCl, 15min at 100°C) to yield Hyp-Lys and Ser-Hyp. The peptide Ser-Hyp₄ also had an electrophoretic mobility at pH 1.9 identical with that of the fourth member of the Ser-Hyp_n homologous series described previously (Lamport, 1971). As the peptide S_2A_6 was isolated from a tryptic digest, lysine is *C*-terminal. The complete sequence of peptide S_2A_6 is therefore Ser-Hyp-Hyp-Hyp-Hyp-Ser-Hyp-Lys. Thus two serine residues are present, one of them as the *N*-terminus, the other internally situated.

Attachment of galactose to peptide S_2A_6

The galactose content measured by the use of Galactostat after hydrolysis of the peptide in trifluoroacetic acid, by the cysteine- H_2SO_4 method and by g.l.c. of the alditol acetates, was consistently two residues/molecule of peptide. No other sugars were detected either by paper chromatography or by g.l.c. after conversion into the alditol acetates. The only groups available for glycosidic attachment of galactose to the peptide are the hydroxyl groups of serine

Table 1. *Partial acid hydrolysis of peptide S_2A_6*

The analysis is expressed as molar ratios uncorrected for losses during acid hydrolysis. The original sample of glycopeptide S_2A_6 contained 1590 μ g of hydroxyproline. n.d., Not determined.

Fractions	Analysis			<i>N</i> -Terminus	Deduced sequence	Total Hyp in fraction (μ g)	Yield (% of theoretical)
	Ser	Hyp	Lys				
Major peptides							
7-12	1.0	4.0	0	Ser	Ser-Hyp ₄	652	51
126-131	0.9	1.0	1.3*	Ser	Ser-Hyp-Lys†	162	51
Minor peptides							
3-6	1.8	4.0	0	n.d.	n.d.	47	4
13-17	0.8	3.0	0	Ser	Ser-Hyp ₃	101	11
21-24					Free Hyp	34	—
28-32	0.9	4.0	0	n.d.	n.d.	17	—
45-51	0.8	1.0	0	n.d.	n.d.	7	1.1
52-56	0.9	3.0	0	Hyp	n.d.	17	—
57-61	1.9	4.0	0	Ser	Probably: Ser-Hyp ₄ -Ser	17	1.3
62-68	1.7	5.0	1.0	Ser	Ser-Hyp ₄ -Ser-Hyp-Lys‡	179	11
108-118	1.0	3.0	1.0	n.d.	n.d.	55	—
119-122	1.6	5.0	1.2	Ser	Ser-Hyp ₄ -Ser-Hyp-Lys	45	3.5
134-137	0	1.0	1.0	Hyp	Hyp-Lys	5	1.5
					Total Hyp recovered	1338	84

* Contaminated by a small amount of free lysine which is also eluted in this area of the Aminex A-5 chromatogram shown in Fig. 1.

† On further partial acid hydrolysis (6M-HCl, 15min at 100°C) this peptide gave Hyp-Lys and Ser-Hyp.

‡ Previous experiments (D. T. A. Lamport, L. Katona & S. Roerig, unpublished work) indicate that this peptide probably contains one galactosyl residue. After amino acid analyses and *N*-terminus determination insufficient material remained to confirm the presence of galactose.

and hydroxyproline, the amino groups of lysine and the *N*-terminal serine residues and the carboxyl group of the *C*-terminal lysine. The electrophoretic mobility at pH 6.5 was 0.27 relative to aspartic acid. This indicates a net positive charge of 1 for a peptide of this size and composition, hence a free carboxyl terminus. The origin of the peptide from a tryptic digest also made it unlikely that the carboxyl group or either of the amino groups was involved in the sugar linkage. Earlier treatment of the cell walls at pH 1 for 1 h at 100°C removed all the sugar substituents on the hydroxyproline hydroxyl groups. This was verified by finding that S₂A₆ (as well as other galactoglycopeptides not described here) contained no hydroxyproline glycosides when tested by the method of Lamport & Miller (1971). Thus the serine hydroxyl group remains as the only reasonable point of attachment. However, the peptide did not show the expected conversion of serine into alanine when β -elimination was attempted by using mild conditions (0.05M-NaOH at 4°C for 18 h in the presence of 0.2M-NaBH₄). Elimination did not occur under these mild conditions even after the *N*-terminus had been substituted by maleylation or 3-carboxypropionylation in an effort to overcome the much higher resistance of *N*-terminal serine *O*-glycosides to β -elimination.

Increasing the severity of the reaction conditions ultimately led to complete elimination of galactose with a concomitant loss of serine. For example, maleylated peptide at room temperature in the pre-

sence of NaOH and NaBH₄ showed significant conversion of serine into alanine after 24 h and 48 h (Table 2, Expts. 2, 3, 4). In Expt. 2 (Table 2) there was a good correlation between disappearance of serine and loss of galactose from the peptide. Even after 48 h at room temperature (Table 2, Expt. 4) only about 60% of the serine disappeared. However, raising the temperature to 50°C led to virtually complete disappearance of serine within 5 h (Fig. 2), whereas 45% of the theoretical yield of the remaining alanine appeared in the reaction mixture. Repurification of the eliminated peptide by gel filtration on Sephadex G-25 gave a galactose-free peptide representing 37% of the original peptide. The purified galactose-free peptide contained 90% of the theoretical yield of alanine. Essentially similar results were obtained when the peptide (S₂A₆) was 3-carboxypropionylated (Table 2, Expt. 5), with the exception that lysine yields were invariably decreased. After unmodified peptide S₂A₆ (i.e. with free *N*-terminus) was treated with NaOH-NaBH₄ for 5 h at 50°C (Table 2, Expt. 1) slightly more than one residue of serine disappeared, indicating that of the two serine residues one underwent an elimination reaction more readily than the other.

Treatment of unmodified peptide S₂A₆ with 50 μ l of 0.25M-NaOH-1.0M-NaBH₄ under N₂ for 5 h at 50°C followed by acetylation and g.l.c. gave only one product. This was identified as hexa-acetylgalactitol. The yield of hexa-acetylgalactitol was 53.5% of the

Table 2. *Effect of alkali on Ser-Hyp₄-Ser-Hyp-Lys(Gal₂) in the presence of NaBH₄*

Compositions are expressed as amino acid residues relative to hydroxyproline. Values are not corrected for losses during acid hydrolysis. In Expt. 2 the content of galactose was 2.0 residues before elimination and 1.3 residues after elimination. Values are not corrected for losses during acid hydrolysis.

Expt. no.	Peptide pretreatment	Elimination conditions	Composition					
			Before elimination			After elimination		
			Hyp	Ser	Lys	Hyp	Ser	Ala
1	None	0.2M-NaOH 0.2M-NaBH ₄ 5h, 50°C	5	1.8	1.0	5	0.65	0.32
2	Maleylated	0.2M-NaOH 0.2M-NaBH ₄ 24h, 25°C	5	1.7	1.0	5	0.95	0.4
3	Maleylated	0.2M-NaOH 1.0M-NaBH ₄ 24h, 25°C	5	1.7	1.0	5	1.2	0.07
4	Maleylated	0.2M-NaOH 1.0M-NaBH ₄ 48h, 25°C	5	1.7	1.0	5	0.7	0.2
5	3-Carboxypropionylated	0.2M-NaOH 0.2M-NaBH ₄ 5h, 50°C	5	1.5	0.5	5	0.09	0.55

total galactose in the peptide, which in conjunction with the previous experiment (Table 2, Expt. 1 above) indicates that a single residue of galactose was eliminated from one of the serine residues. The experiment described above (Fig. 2) in which both serine residues underwent an elimination reaction to yield a galactose-free peptide supports the further inference that each serine residue is *O*-galactosylated.

In an effort to obtain a better conversion of serine than that obtained by the NaOH-NaBH₄ technique, we considered β -elimination in the presence of Na₂SO₃ (Harbon *et al.*, 1968). Significant conversion of serine into cysteic acid occurred when elimination of 3-carboxypropionylated peptide took place in the presence of Na₂SO₃ at room temperature, and an almost theoretical conversion occurred on increasing the temperature to 50°C (Table 3, Expt. 3). On the

correct the new peptide [Ser-Hyp-Hyp-Hyp-Hyp-Cys(O₃H)*-Hyp-Lys] produced by sulphonation of peptide S₂A₆ under alkaline conditions should now contain one residue of galactose attached to the *N*-terminal serine. This possibility was tested by treatment of peptide S₂A₆ with 1 M-Na₂SO₃ in 0.2 M-NaOH for 3 h at 50°C followed by gel filtration (Sephadex G-25) to purify the new peptide. The results (Table 4) show that per molecule the new peptide contained 1.0 residue of serine, 1.0 residue of galactose and 0.8 residue of cysteic acid. Treatment of this peptide with acrylonitrile by Fletcher's (1966) method confirmed that the remaining serine residue was predominantly *N*-terminal (Table 4).

The structure of glycopeptide S₂A₆ as isolated by tryptic digestion of acid-stripped cell walls is therefore:

other hand unmodified peptide (i.e. free *N*-terminus) treated with NaOH-Na₂SO₃ at 50°C gave 1.1 residues of cysteic acid and 0.7 residue of serine (Table 3, Expt. 1). When this sulphonated peptide was treated

which, in view of the fact that most of the cell-wall-bound hydroxyproline is *O*-substituted by tetra-arabinose, may have the following structure in the wall:

with acrylonitrile at alkaline pH followed by acid hydrolysis and amino acid analysis serine could no longer be detected (Table 3, Expt. 2). Therefore sulphonation of the unmodified glycopeptide converted the internal serine residue into cysteic acid, and the *N*-terminal serine residue was essentially stable under the elimination conditions used.

If the interpretation of previous experiments is

Stability of galactosylserine in N₂H₄ and strong alkali

Peptide S₂A₆ was heated with anhydrous N₂H₄ for 10 h at 100°C. The hydrazinolysate was hydrolysed with 6 M-HCl for 18 h at 105°C. Amino acid analysis (Table 5) showed that nearly all the serine was lost. One new peak was seen after amino acid analysis. It

* Abbreviation: Cys(O₃H), cysteic acid.

Fig. 2. Time-course of the β -elimination by alkali of galactose residues from maleylated Ser-Hyp₄-Ser-Hyp-Lys(Gal₂) in the presence of NaBH₄.

Tomato cell-wall glycopeptide S₂A₆ was maleylated and then incubated in a microvial containing 1 ml of 1.0M-NaBH₄ in 0.2M-NaOH at room temperature. Samples of 10 μ l each (containing 22 nmol of peptide) were taken after 24 h and 48 h, hydrolysed directly in 200 μ l of 6M-HCl and analysed for amino acids (Table 2, Expts. 3 and 4). After the 48 h sample was taken the temperature was increased to 50°C and samples were taken over the next 5 h and analysed as above. The Figure shows the disappearance of serine (●) and appearance of alanine (□) during this treatment at 50°C. Results are expressed as residues of serine and alanine relative to five residues of hydroxyproline. After the 5 h sample had been taken the remaining peptide was neutralized with acetic acid and repurified by gel filtration on a column (120 cm $\times$ 0.6 cm) of Sephadex G-25. The repurified eliminated peptide (37% yield) had the following composition: Hyp₅-Ser_{0.2}-Ala_{1.8}-Lys_{0.8}. Galactose was absent.

corresponded to *cis*-4-hydroxy-D-proline and showed that 25% of the hydroxyproline was racemized, an indication that the hydrazinolysis conditions were sufficiently basic for the elimination of galactose from the serine residues. Judging from the loss of serine (Table 5, Expt. 2) the galactosylserine linkage is also unstable in the strong alkaline conditions (17.5% NaOH) normally used for the extraction of hemicellulose. These results together with our isolation of alkali-stable hydroxyproline hetero-oligosaccharides from *Chlamydomonas* (Miller *et al.*, 1972), but never from higher plants, suggest that the neutral glycopeptide B (reported to contain only hydroxyproline, arabinose and galactose) isolated by hydrazinolysis of sycamore cell walls (Heath & Northcote, 1971) could be an artifact due to incomplete separation of neutral arabinogalactan from a (hydroxypropyl)_n-(arabinose)_n glycopeptide.

Extent of serine glycosylation in the cell wall and the attachment of polysaccharide to extensin

As expected from the results obtained with peptide S₂A₆ (Table 5), cell walls treated with alkali (Table 6) or N₂H₄ (Table 6) also lost appreciable amounts of serine. In control experiments where trypsin and albumin (Lamport, 1972) and glucagon (Table 7) were hydrazinolysed and then subjected to acid hydrolysis serine residues were not lost. These results indicate that at least 50% of the serine residues were glycosylated in sycamore and tomato cell walls.

Our observation that treatment of cell walls and peptide S₂A₆ with 17.5% NaOH or N₂H₄ leads to a loss of serine residues therefore refutes the claim (Heath & Northcote, 1971) that 'The extraction of the (hydroxyproline-rich) glycopeptides was achieved without cleavage of glycosyl bonds, so that the glycoprotein cannot act as a covalent cross-link be-

Table 3. Effect of alkali on Ser-Hyp₄-Ser-Hyp-Lys(Gal₂) in the presence of Na₂SO₃

Compositions are expressed as amino acid residues relative to hydroxyproline. Values are not corrected for losses during acid hydrolysis.

Expt. no.	Peptide pretreatment	Elimination conditions	Composition					
			Before elimination			After elimination		
			Hyp	Ser	Lys	Hyp	Ser	Cys(O ₃ H)
1	None	0.25M-NaOH 1.0M-Na ₂ SO ₃ 5h, 50°C	5	1.8	1.0	5	0.7	1.1
2	None	As in Expt. 1, but then cyanoethylated	5	1.8	1.0	5	0.0	0.8
3	3-Carboxypropionylated	0.25M-NaOH 1.0M-Na ₂ SO ₃ 5h, 50°C	5	1.5	0.5	5	0.0	1.6

Table 4. *Reisolation and analysis of peptide S₂A₆ after treatment with alkaline sulphite*

A sample of peptide S₂A₆ containing 500 µg of hydroxyproline was treated at 50°C for 3 h in 100 µl of 0.2M-NaOH containing 1.0M-Na₂SO₃. Separation of the reaction mixture on Sephadex G-25 gave the sulphonated peptide in 87% yield. In control experiments these conditions gave non-specific sulphonation of 12% of the serine residues of glucagon (Table 7). Compositions are expressed as residues relative to hydroxyproline. Values are not corrected for losses during acid hydrolysis.

Expt. no.	Conditions	Compositions				
		Hyp	Ser	Cys(O ₃ H)	Lys	Gal
1	Before alkali treatment	5	1.9	0	1.0	1.84
2	After alkali treatment and gel filtration on Sephadex G-25	5	1.0	0.8	1.1	1.02
3	As Expt. 2, but after further treatment with acrylonitrile	5	0.3	0.9	0	—

Table 5. *Effect of N₂H₄ and alkali on Ser-Hyp₄-Ser-Hyp-Lys(Gal₂)*

Compositions are expressed as amino acid residues relative to hydroxyproline. At the concentration of NaOH used in Expt. 2, the addition of NaBH₄ did not result in the appearance of alanine.

Expt. no.	Conditions	Composition					
		Before treatment			After treatment		
		Hyp	Ser	Lys	Hyp	Ser	Lys
1	N ₂ H ₄ , 10h at 105°C	5	1.9	1.1	5	0.1	1.1
2	17.5% (w/v) NaOH, 10h at 23°C	5	1.9	1.1	5	0.8	1.1

Table 6. *Effect of N₂H₄ and NaOH on the amino acid composition of cell walls isolated from suspension cultures*

The results are given as residues relative to 30 residues of hydroxyproline. After treatment of cell walls with NaOH the cell walls were dialysed to remove the NaOH before acid hydrolysis.

	Tomato			Sycamore		
	Untreated	N ₂ H ₄ , 10h at 105°C	4.4M-NaOH, 10h at 23°C	Untreated	N ₂ H ₄ , 10h at 105°C	4.4M-NaOH, 10h at 23°C
Hyp	30	30	30	30	30	30
Asp	12	9	7	9	8	10
Thr	7	1	4	5	3	5
Ser	15	5	8	14	7	11
Glu	9	13	8	10	11	11
Pro	9	8	7	5	10	8
Gly	10	16	5	7	14	7
Ala	9	8	6	7	7	7
Val	9	10	7	8	11	11
½-Cys	0	0	0	0	0	0
Met	1	8*	2	1	13*	1
Ile	4	5	3	4	4	4
Leu	8	8	6	6	7	7
Tyr	1	3	3	2	4	4
Phe	3	2	3	3	5	4
Lys	12	14	10	10	17	11
His	2	2	3	2	3	3
Arg	2	0	3	2	0	3

* This appears to be an unknown ninhydrin-reactive peak, which is eluted in the methionine area, and is expressed as nor-leucine equivalents.

Table 7. *Effect of alkaline sulphite and N₂H₄ on glucagon*

The results are given as residues relative to three residues of glutamic acid. Results for untreated glucagon were taken from *Handbook of Biochemistry: Selected Data for Molecular Biology*, 2nd edn. (1970) (Sober, H. A., ed.), p. C262, Chemical Rubber Co., Cleveland. For the N₂H₄ treatment, anhydrous N₂H₄ (200 µl) was heated with 200 µg of glucagon in a sealed microvial. After removal of N₂H₄ the hydrazinolysate was hydrolysed for 18 h in 6M-HCl at 105°C. With the NaOH-Na₂SO₃ treatment, after treatment of 200 µg of glucagon with 100 µl of alkaline sulphite for 3 h at 50°C the reaction was stopped by addition of 50 µl of 6M-HCl followed by evaporation in a stream of N₂. After hydrolysis for 18 h at 105°C in 200 µl of 6M-HCl the hydrolysate was evaporated almost to dryness in a stream of N₂. The amino acid analyser starting buffer (100 µl, pH 2.875) was immediately added. Control experiments in which the alkaline sulphite treatment was stopped at zero time show that when the above procedures were followed and freshly prepared Na₂SO₃ was used, sulphoserine was not produced.

	Untreated	Treatment	
		N ₂ H ₄ , 10h at 105°C	0.2M-NaOH- 1.0M-Na ₂ SO ₃ , 3h at 50°C
Cys(O ₃ H)	0	0	0.5
Asp	4	3.9	4.0
Thr	3	2.1	2.6
Ser	4	3.7	3.3
Glu	3	3.0	3.0
Pro	0	0	0
Gly	1	1.6	1.3
Ala	1	1.0	0.9
Val	1	0.9	0.9
Met	1	1.0	1.0
Leu	2	1.9	1.9
Tyr	2	1.9	1.8
Phe	2	1.9	1.8
Lys	1	1.1	1.1
His	1	0.9	0.8
Arg	2	0	1.9

tween the major polysaccharides of the wall'. Clearly the galactosylserine linkage is sensitive to alkali and in this respect agrees with the earlier inference (Lamport, 1970) of an alkali-labile linkage region between saccharide and peptide components of extensin. The alkali-labile galactosylserine is therefore a better candidate for the role of polysaccharide attachment than are the alkali-stable hydroxyproline arabinosides. However, the precise site of

polysaccharide attachment to extensin requires the demonstration of rather more than the attachment of single galactose residues to serine, although the monomeric attachment shown here might represent that portion of polysaccharide not cleaved by the acidic pretreatment required for tryptic degradation of the walls. The demonstration of another glycopeptide linkage in extensin is therefore additional evidence which is increasingly in favour of the view that the primary cell wall of higher plants exists as a protein-glycan network (Lamport, 1962, 1970) where a '... network of protein, pectic polymers and xyloglucan serves to cross-link the cellulose fibers of the wall' (Keegstra *et al.*, 1972).

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