A Hydroxyproline-Containing Glycopeptide Released from the Walls of Sycamore Tissue-Culture Cells by Hydrazinolysis

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Degradation of the sycamore cell wall by hydrazinolysis yielded soluble glycopeptides. The peptide portion of the predominant glycopeptide was shown to be cyclic (4-*trans*-hydroxy-L-prolyl)-4-*trans*-hydroxy-L-proline. From this structure and the composition of the major glycopeptide it was deduced that only short oligosaccharides are attached to the hydroxyprolyl residues of the wall glycoprotein. Detailed evidence for the structure of the peptide is given in a supplementary paper that has been deposited as Supplementary Publication SUP 50020, at the National Lending Library for Science and Technology, Boston Spa, Yorks. LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1973) 131, 5.

The presence of protein, rich in 4-*trans*-hydroxy-L-proline (subsequently referred to as hydroxyproline), in the walls of plant cells has been known for some time (Lamport & Northcote, 1960; Dougall & Shimbayashi, 1960). The protein has been shown to carry oligosaccharide side chains, containing arabinose and galactose (Lamport, 1969*a*, 1970; Heath & Northcote, 1971), and its method of synthesis has been widely studied (Chrispeels, 1970; Brysk & Chrispeels, 1972; Dashek, 1970; Karr, 1971). Fractionation of sycamore cell walls demonstrated that, under mild conditions, the hydroxyproline-rich protein of the cell wall remained in association with the cellulose of the wall in an insoluble complex (Heath & Northcote, 1971). Treatment of the complex with anhydrous hydrazine-hydrazinium sulphate yielded glycopeptide material from which the major component could be separated by gel filtration and paper electrophoresis.

Experimental

Tissue

Sycamore (*Acer pseudoplatanus*) tissue was grown in suspension culture, as described by Stoddart *et al.* (1967). Cells were harvested by centrifugation and washed with water.

Methods

Preparation of radioactive glycopeptides. The labelling of tissues with L-[1-¹⁴C]arabinose and L-[U-³H]-

proline, the preparation and fractionation of walls, and the technique of hydrazinolysis with anhydrous hydrazine containing 1.5% (w/v) hydrazinium sulphate have been described previously (Heath & Northcote, 1971). Hydrazinolysates of either whole walls or the insoluble cellulose complex from fractionated walls were dried and extracted with water. The aqueous extracts were fractionated by gel filtration on a column (3 cm × 40 cm) of Sephadex G-25.

Dilute acid treatment. The material was dissolved in 0.1 M-HCl and heated at 100°C for 1 h in a sealed tube (Lamport, 1969*b*) in an oil bath. The acid was removed by evaporation under reduced pressure. This procedure will split arabinosyl bonds without damage to the hydroxyprolyl peptide.

Synthesis of cyclic (4-*trans*-hydroxy-L-prolyl)-4-*trans*-hydroxy-L-proline. The method of D'Alagni & Pispisa (1969) was used to convert hydroxyproline methyl ester hydrochloride into the cyclic dipeptide. The product was recrystallized from methanol-water (1:1, v/v).

Determination of N-terminal amino acid residue. The phenyl isothiocyanate method of Edman was used (Edman & Begg, 1967; Gray, 1967) on a radioactive sample. The reaction product was extracted sequentially with benzene, pyridine and aq. 50% (v/v) pyridine, and the radioactivity of each extract was determined on paper.

Determination of hydroxyproline. The method of Leach (1960) was used.

Paper electrophoresis, paper chromatography and scintillation counting. These were carried out as previously described (Heath & Northcote, 1971).

Gas-liquid chromatography. Samples were run as trimethylsilyl ether derivatives on a 2.1 m (7 ft) SE 54

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column at 228°C in a Pye Series 104 gas chromatograph with He at 45 ml·min⁻¹ as carrier gas.

Mass spectrometry. The output from the gas chromatograph was passed through a separator to an AEI MS902 mass spectrometer with an ion source temperature of 220°C and an ionizing potential of 70 eV.

Infra-red spectrophotometry. The sample was analysed as a Nujol mull in a Perkin Elmer 257 grating i.r. spectrophotometer.

Results

Preparation of glycopeptide material from whole walls or cellulose complex

Gel filtration of hydrazinolysates on Sephadex G-25 yielded two peaks of glycopeptide material (i.e. containing both ¹⁴C and ³H radioactivity). Paper electrophoresis (pH 2) of material from the first (i.e. higher-molecular-weight) glycopeptide peak yielded a component with zero mobility that accounted for most of the radioactivity of the peak, and which was termed component B (Heath & Northcote, 1971).

Preparation of B peptide

Dilute acid treatment (pH 1, 100°C, 1 h) followed by paper chromatography yielded all the ³H radioactivity of component B as a single fast-running species, B peptide (*R*_{Glc} 2.2), which consisted only of hydroxyproline (Heath & Northcote, 1971). The B peptide was eluted from the chromatogram with water.

N-terminus

After B peptide material had been subjected to a cycle of Edman degradation, no radioactivity could be extracted from the reaction mixture with benzene, although the radioactive material was still soluble in pyridine. This indicated that no phenylthiohydantoin had been formed and suggested that B peptide does not have a free amino group.

Synthetic cyclic (4-trans-hydroxy-L-prolyl)-4-trans-hydroxy-L-proline

Hydrolysis and assay of a sample of the synthetic product indicated that it contained at least 94% hydroxyproline. The i.r. spectrum was consistent with a cyclic dipeptide as it lacked the secondary amine–amide absorption owing to N–H bending at around 1520 cm⁻¹ but contained a tertiary amide absorption owing to C=O stretching in the 1650 cm⁻¹ region (Williams & Fleming, 1966). The product melted between 240° and 245°C (uncorr.), with charring (Kapfhammer & Matthes, 1934).

Comparison of cyclic (4-trans-hydroxy-L-prolyl)-4-trans-hydroxy-L-proline with B peptide

A sample of the synthetic product was subjected to paper electrophoresis at pH 2 and a section (3 cm long), corresponding to the position of B peptide, was excised from the electrophoretogram; 95% of the sample was recovered from this section. Another sample was chromatographed on paper and a similar section corresponding in position to B peptide was excised. This section contained 84% of the applied sample. G.l.c. of the trimethylsilyl ether derivative of B peptide yielded a single peak with a retention time of 15.4 min. The derivative of the synthetic product gave a major peak with a retention time of about 15.7 min, and a minor peak at 13.8 min. The mass spectrum of material from the major peak was closely similar to that of the B peptide derivative and consistent with di-*O*-trimethylsilyl-cyclic (4-*trans*-hydroxy-L-prolyl)-4-*trans*-hydroxy-L-proline. The minor peak gave a similar but different spectrum and was probably a different epimer of the cyclic dipeptide derivative. The g.l.c. and mass spectrometry are described and discussed in the Supplementary Publication (SUP 50020).

Discussion

The high chromatographic mobility of B peptide was an unexpected feature and a further anomaly was the zero electrophoretic mobility at pH 2 and pH 6.5 (Heath & Northcote, 1971). The absence of a detectable *N*-terminal amino acid and reported behaviour of imino acid-containing dipeptides (Smith & Bergmann, 1944; Lampiaho *et al.*, 1972) suggested that B peptide might be cyclic (4-*trans*-hydroxy-L-prolyl)-4-*trans*-hydroxy-L-proline (Fig. 1.). The g.l.c. and mass spectrometry of the trimethylsilyl ether derivatives of B peptide and of synthetic cyclic dipeptide confirmed that B peptide was (4-*trans*-hydroxy-L-prolyl)-4-*trans*-hydroxy-L-proline. The evidence of paper chromatography and electrophoresis and the absence of an *N*-terminus in B peptide demonstrated that it was a cyclic dipeptide.

Fig. 1. Cyclic (4-*trans*-hydroxy-L-prolyl)-4-*trans*-hydroxy-L-proline

It is likely that cyclization occurred when a dipeptide hydrazide was released from the protein during hydrazinolysis. Tetra-hydroxyprolyl sequences have been found in tryptic peptides from wall protein (Lamport *et al.*, 1971). Imino acid dipeptides show a marked tendency to cyclize (Abderhalden & Nienburg, 1933; Lampiaho *et al.*, 1972); with a hydrazide the reaction is probably even easier, being effectively a transamination rather than amide formation. The lactam linkages in the cyclic dipeptide should be more stable to hydrazine than the linear amides of the protein (Bruce & McMahon, 1972).

In component B, the molar proportions of hydroxyproline/arabinose/galactose are 2:7:11 and every hydroxyprolyl residue carries a sugar chain (Heath & Northcote, 1971). If the peptide portion, B peptide, contains only two residues of hydroxyproline then the longest side chain possible contains seventeen sugars, although the more even distribution of eight-ten units/hydroxyproline seems more reasonable. As component B can be prepared from whole walls by hydrazinolysis only, and is the largest glycopeptide so produced, there can be no large polysaccharides connected to the protein through the hydroxyproline, as has been suggested (Lamport, 1969*a*, 1970).

The action of anhydrous hydrazine-hydrazinium sulphate on the hydroxyproline-rich glycoprotein of the sycamore cell wall is to split out the hydroxyproline as small glycopeptides. Where a Hyp-Hyp sequence is released, this cyclizes and cyclic (4-*trans*-hydroxy-L-prolyl)-4-*trans*-hydroxy-L-proline, each hydroxyprolyl residue bearing a carbohydrate side chain, is the major product of hydrazinolysis, forming the first (i.e. higher-molecular-weight) glycopeptide peak on gel filtration. As the carbohydrate side chains are not subject to degradation during hydrazinolysis by this technique (Matushima & Fujii, 1957; Kaverzneva & Tsi, 1961; Yosizawa & Sato, 1962; Yosizawa *et al.*, 1966; Aston *et al.*, 1968; Heath & Northcote, 1971) the results indicate that only small oligosaccharides are connected to the hydroxyprolyl residues of the wall glycoprotein.

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