Isodityrosine, a new cross-linking amino acid from plant cell-wall glycoprotein

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1. Cell-wall hydrolysates from calli of all higher plants tested contained a new phenolic amino acid for which the trivial name isodityrosine is proposed. Isodityrosine was shown to be an oxidatively coupled dimer of tyrosine with the two tyrosine units linked by a diphenyl ether bridge. 2. The amount of isodityrosine in sodium dodecyl sulphate-insoluble cell-wall preparations was proportional to the amount of hydroxyproline. 3. Acidified chlorite split the diphenyl ether bridge of isodityrosine, and concomitantly solubilized the cell-wall glycoprotein. 4. Dithiothreitol inhibited isodityrosine synthesis *in vivo*, and suppressed in parallel the covalent binding of newly synthesized protein in the cell wall. 5. It is suggested that isodityrosine is an inter-polypeptide cross-link responsible for the insolubility of plant cell-wall glycoprotein.

Plant cell walls contain a highly insoluble hydroxyproline-rich glycoprotein ('extensin') that co-purifies with α -cellulose (Lamport & Northcote, 1960; Heath & Northcote, 1971; Monro *et al.*, 1976; Esquerré-Tugayé & Mazau, 1981). Its biological role is the subject of much speculation (for review see Esquerré-Tugayé & Mazau, 1981). The nature of its presumed covalent binding in the wall is also controversial (Keegstra *et al.*, 1973; Monro *et al.*, 1976; Pope, 1977), and is investigated in the present study as a contribution to the elucidation of its role.

Anhydrous hydrogen fluoride completely depolymerizes the cell-wall carbohydrates (including cellulose) without solubilizing the protein (Mort & Lamport, 1977). This shows that cross-linking with polysaccharides is not responsible for its insolubility. However, the glycoprotein can be solubilized, with minimal degradation, in acidified chlorite (O'Neill & Selvendran, 1980), a reagent traditionally used for de-lignification because it cleaves phenolic polymers. This suggests that the protein is held in the wall by aromatic cross-links similar to those of lignin.

In the present paper I report the widespread occurrence in plant cell-wall protein of a new phenolic amino acid that could fulfil such a cross-linking role. Its structure implies post-translational biosynthesis via the peroxidase-catalysed coupling of two tyrosine units. Such coupling between protein molecules within the cell wall might limit wall extensibility and therefore plant growth.

Materials and methods

Radiochemicals were from Amersham International. Poly-L-tyrosine (M_r 40 000–100 000) was from Sigma Chemical Co. Folin–Ciocalteu phenol reagent was purchased from BDH Chemicals.

Cell-suspension cultures and calli of potato (*Solanum tuberosum* L.) were maintained as described by Murray & Northcote (1978). Other cultures were maintained under standard conditions. For isolation of cell walls, the cells were harvested by filtration on muslin, and plunged into boiling 3% (w/v) sodium dodecyl sulphate containing 1% (v/v) 2-mercaptoethanol and 1% (w/v) NH_4HCO_3 . The suspension was stirred at 100°C for 20 min and filtered on muslin, and the extraction was repeated at least twice more. The final (cell-wall) residue was rinsed free of detergent with water, and freeze-dried. Hydrolysis was performed with less than 4 mg of cell walls/ml of 6 M-HCl (containing 10 mM-phenol) in a sealed tube at 110°C for 24 h. The HCl was removed *in vacuo* with a Speed-Vac evaporator (Savant Instruments).

For assay of non-radioactive isodityrosine, the hydrolysate was electrophoresed at pH 2.0 (solvent 1; 5 kV for 30 min) followed by chromatography in the second dimension in solvent 4. The dried paper was sprayed almost to saturation with Folin–Ciocalteu reagent, and treated with NH_3 vapour until the yellow background was decolorized. The paper was dried again, and the blue spot due to isodityrosine (see Table 1) was eluted with 2.5 M-

NaOH and assayed spectrophotometrically at 740 nm. Quantities of isodityrosine were estimated on the assumption that the molar colour yield was equal to that of tyrosine.

For analysis of hydroxyproline, samples were electrophoresed at pH 2.0 (solvent 1; 5 kV for 45 min) and the dried paper was stained with isatin/ninhydrin reagent (Kolor & Roberts, 1957). The dry paper was washed in water to decolorize the background, and re-dried, and the pink spot due to hydroxyproline was eluted with acetone/ethanol (1:2, v/v) and assayed spectrophotometrically at 515 nm. Known amounts of authentic 4-hydroxy-L-proline were processed in parallel.

Radioactive hydrolysates were briefly electrophoresed at pH 2.0 (solvent 1; 5 kV for 15 min), and radioactivity was eluted from a broad zone of the electrophoretogram that included all the amino acids but excluded acidic and neutral compounds. The eluate was chromatographed in solvent 3, and strips of the paper were assayed for radioactivity.

Chromatography and electrophoresis were performed on Whatman no. 1 paper (except where otherwise stated). High-voltage electrophoretograms were run over a path length of approx. 50 cm, in tanks of 'white spirit' at 20–30°C. Low-voltage runs (200 V) were over a path length of 21 cm on paper trapped between air-cooled glass plates (5–15°C). Solvents used were (all compositions by vol.): 1, formic acid/acetic acid/water (1:4:45) (pH 2.0); 2, acetic acid/pyridine/water (1:33:300) (pH 6.5); 3, butan-1-ol/acetic acid/water (15:3:5); 4, propan-2-ol/aq. 17% NH₃ (4:1); 5, propan-1-ol/aq. 25% NH₃ (7:3); 6, benzene/acetic acid (9:1); 7, 90% (v/v) formic acid/acetic acid/water (134:100:268) (pH ~0.6).

Strips (1 cm × 4 cm) from radioactive chromatograms were assayed for radioactivity without elution, by liquid-scintillation counting in 2 ml of 0.5% (w/v) 2,5-diphenyloxazole in toluene. The efficiency was approx. 20%.

Authentic dityrosine was synthesized by a modification of the method of Gross & Sizer (1959). L-Tyrosine (25 mM, pH adjusted to 9.4 with NaOH) was incubated with horseradish peroxidase [0.1 mg/ml; RZ (A_{403}/A_{275} ratio) = 1.6] at 30°C for 22 h. The product was purified on Sephadex LH-20 in 35% (v/v) methanol, followed by chromatography in solvent 3. Isodityrosine was purified from hydrolysates of potato callus cell walls on Sephadex G-25 followed by chromatography in solvents 3 and 4 and electrophoresis at pH 2.0 (solvent 1; 5 kV for 30 min). The course of the purification was monitored by the staining of narrow strips with Folin–Ciocalteu reagent and ninhydrin. Purified isodityrosine was eluted with 5 M-NH₃, freeze-dried, and stored at –20°C.

Amino acids were partially dinitrophenylated by a

modification of the method of Andersen (1963). To 10 µl of amino acid solution (approx. 0.1% in 5% Na₂CO₃) was added 2.5 µl of 10% (w/v) 1-fluoro-2,4-dinitrobenzene in acetone. The reaction was stopped after 0.1, 1, 5 or 25 min at 20°C by extraction of the remaining fluorodinitrobenzene into 1 ml of ice-cold diethyl ether. The organic phase was rejected, and the aqueous phase was supplemented with 20 µl of water and washed with a further 1 ml of ether. Residual traces of ether were removed by evaporation, and the aqueous solution was shaken with 5 µl of acetic acid plus 10 µl of acetone to neutralize the Na₂CO₃ and dissolve the yellow product. The whole reaction mixture was then electrophoresed at pH 0.6 (solvent 7; 200 V for 4 h). The yellow spots were recorded by photography through a blue filter, and the paper was finally stained with ninhydrin (0.2% in acetone).

Amino acids were acetylated in aq. 50% (v/v) acetic anhydride at 20°C for 30 min, and then dried *in vacuo*. For mass spectrometry, the samples (in solution in glycerol) were subjected to fast atom bombardment in a Kratos MS-50 instrument fitted with a standard Kratos FAB source (Barber *et al.*, 1981; Williams *et al.*, 1981). The negative ions generated were monitored for *m/z*.

Results and discussion

Occurrence and origin of isodityrosine

Acid hydrolysates of potato callus cell walls revealed two positively charged phenols on paper electrophoresis at pH 2.0. The slower-migrating one was tyrosine; the other shared many properties with dityrosine (Fig. 1a and Table 1), but was not fluorescent, and is here called isodityrosine. Wall hydrolysates from ten other species all contained isodityrosine in amounts proportional to the amount of hydroxyproline (isodityrosine/hydroxyproline molar ratio approx. 1:15) (Fig. 1c). This suggests that isodityrosine is an integral component of the insoluble glycoprotein. Since the protein contains about 35 mol of hydroxyproline/100 mol of residues (O'Neill & Selvendran, 1980), the data indicate 2.3 mol of isodityrosine/100 mol of residues. Isodityrosine might be the uncharacterized 'tyrosine derivative' reported in wall glycopeptide sequences (Lampert, 1977).

In potato callus, isodityrosine could be detected only in the cell-wall fraction. This was equally true whether the phenolic amino acids were stained with Folin–Ciocalteu reagent (results not shown) or detected by ¹⁴C-labelling (Figs. 2b and 2f).

Evidence was obtained that the isodityrosine was not formed as an artifact: (1) hydrolysis of cell walls (10 mg) in the presence of 0.2 mg of tyrosine, polytyrosine or tryptophan yielded no more isodityrosine than did hydrolysis of cell walls alone; (2)

Fig. 1. Chemical structure and natural occurrence of isodityrosine

(a) Structure of dityrosine. (b) Proposed structure of isodityrosine. (c) Isodityrosine and hydroxyproline contents found in hydrolysates of cell walls from calli of 11 plant species. The species tested were: 1, spinach; 2, Scots pine; 3, Virginia creeper (crown gall); 4, rose ('Paul's Scarlet'); 5, peanut; 6, woad; 7, sycamore; 8, potato; 9, tobacco; 10, French bean; 11, parsley.

hydrolysis of cell walls in the presence of tracer concentrations of [^{14}C]tyrosine gave rise to no radioactive isodityrosine (Fig. 2a).

[^{14}C]Tyrosine was, however, readily incorporated into isodityrosine *in vivo* (Fig. 2b), whereas radioactive phenylalanine, cinnamic acid and proline were not (Figs. 2c–2e). Isodityrosine therefore has a close metabolic relationship to tyrosine, and is not a phenol–hydroxyproline complex such as that described by Whitmore (1978).

Structure of isodityrosine

To determine the number of free amino groups in isodityrosine, samples were partially denitro-

phenylated and then electrophoresed at pH 0.6. Dinitrophenylation of an amino group neutralizes it, giving a yellow product of decreased (or zero) electrophoretic mobility. In control experiments, alanine, tyrosine and glutamate gave one immobile, ninhydrin-negative, yellow spot. Dityrosine and lysine both gave two yellow spots: a ninhydrin-positive one with about half the mobility of the parent amino acid, and a ninhydrin-negative one of zero mobility (Fig. 3). Isodityrosine behaved exactly as dityrosine, suggesting that it has two amino groups. Since isodityrosine was electrophoretically neutral at pH 6.5 (Table 1), it is likely also to have two carboxy groups.

The phenolic pK_a of isodityrosine was much closer to that of tyrosine than to that of dityrosine (Table 1). Isodityrosine therefore probably lacks the diphenolic system of dityrosine.

Fast-atom-bombardment mass spectra of acetylated dityrosine gave $[M-1]^-$ ion peaks at $m/z = 401, 443, 485$ and 527 , i.e. dityrosine substituted with 1, 2, 3 or 4 acetyl groups. The four acetylation sites must be the two amino and two hydroxy groups (Fig. 1a). Treated in the same way, isodityrosine gave peaks at $m/z = 443$ and 485 : these would result from an isomer of dityrosine with one acetylation site blocked. A structure that accounts for all the data is shown in Fig. 1(b).

Strong alkaline hydrolysis (4M-KOH, 110°C , 20h, *in vacuo*) of isodityrosine gave tyrosine as the sole Folin–Ciocalteu-positive product detectable chromatographically (systems 3 and 4). This suggests (Fujimoto *et al.*, 1981) that the ether bridge is *ortho* to the free hydroxy group, as shown in Fig. 1(b). (The second expected hydrolysis product, 3,4-dihydroxyphenylalanine, is alkali-labile, giving Folin–Ciocalteu-negative products.) The *meta* compound would have given a second alkali-stable product, 2,4-dihydroxyphenylalanine.

Cross-linking role of isodityrosine

Purified isodityrosine was rapidly degraded (half-life 2–5 min) by 0.3% sodium chlorite (NaClO_2) in 0.12% acetic acid at 57°C (Fig. 4). An initial product was tyrosine, showing that NaClO_2 completely splits isodityrosine. Wall-bound isodityrosine was also degraded by acidified NaClO_2 , which also solubilized wall-bound hydroxyproline-rich polymers (S. C. Fry, unpublished work; O'Neill & Selvendran, 1980). This observation suggests that the glycoprotein is held in the cell wall by inter-chain isodityrosine cross-links.

Is there enough isodityrosine in the cell wall for such a hypothesis to be tenable? If the polypeptide moiety of the glycoprotein before binding in the wall has M_r 25 000, as in the hydroxyproline-rich soluble potato lectin (Allen *et al.*, 1978), then at 2.3 mol of isodityrosine/100 mol of residues there are about

Table 1. *Characteristics of isodityrosine compared with those of dityrosine and tyrosine*

	Tyrosine	Dityrosine	Isodityrosine
Absorption maximum (nm) at pH 1	275	283	273
Absorption maximum (nm) at pH 13	293	317	298
A at $\lambda_{\max.}$ at pH 13/ A at $\lambda_{\max.}$ at pH 1	1.7	1.4	1.1
Phenolic pK_a	10.1	6.7	9.8
U.v. fluorescence* in NH_3 vapour	None	Blue	None
Folin-Ciocalteu reaction* $-NH_3$	None	None	None
Folin-Ciocalteu reaction* $+NH_3$	Blue	Blue	Blue
Ninhydrin reaction*	Violet	Violet	Violet
m_{tyrosine} on electrophoresis at pH 2.0	1.0	1.1	1.1
m_{picrate} on electrophoresis at pH 6.5	0.0	0.0	0.0
R_F in solvent 3	0.34	0.11	0.12
R_F in solvent 4	0.28	0.01	0.02
R_F on silica gel (t.l.c.) in solvent 5	0.45	0.16	0.19
R_F on silica gel (t.l.c.) in solvent 6	0.00	0.00	0.00
$K_{av.}$ on Sephadex G-25 in water†	1.5	N.D.	1.7
$K_{av.}$ on Sephadex LH-20 in 35% methanol†	1.6	1.0	1.0

* Spot tests were performed on Whatman no. 1 paper.

† $K_{av.}$ values are given relative to Blue Dextran ($K_{av.} = 0.0$) and NaCl ($K_{av.} = 1.0$); N.D., not determined.

Fig. 2. *Chromatography of radioactive amino acids recovered from HCl hydrolysates*

(a) L-[U- ^{14}C]Tyrosine ($0.2 \mu Ci$, $0.32 \mu g$) was added to potato cell walls (10 mg dry wt.), which were then hydrolysed; a sample of the crude hydrolysate was chromatographed. [All six chromatograms were run

9 half-isodityrosine residues per molecule. This is clearly sufficient to cross-link the glycoprotein in an extensive three-dimensional network.

Several animal structural proteins, e.g. insect cuticle resilin (Andersen, 1964), contain dityrosine cross-links, as well as the corresponding trimer, trityrosine. Similarly, *Ascaris* cuticles contain isodityrosine, an oxidatively coupled trimer of tyrosine with one biphenyl bridge and one diphenyl ether bridge (Fujimoto *et al.*, 1981). Intermolecular dityrosine cross-links can be generated between a

on Whatman no. 3 paper in freshly prepared solvent 3. —|— and - - -|— indicate the positions of non-radioactive isodityrosine and tyrosine respectively, added to each sample as internal markers and stained after scintillation counting of the radioactivity. The main peaks of radioactivity are identified on the Figure [Idt, isodityrosine]. (b)–(e) Samples (3.3 ml) from an exponentially growing potato cell-suspension culture were incubated aseptically for 18 h in the presence of: (b) $1.0 \mu Ci$ ($1.6 \mu g$) of L-[U- ^{14}C]tyrosine; (c) $1.0 \mu Ci$ ($0.51 \mu g$) of L-[U- ^{14}C]phenylalanine; (d) $1.0 \mu Ci$ ($5.0 \mu g$) of *trans*-[side-chain-3- ^{14}C]cinnamic acid; (e) $0.5 \mu Ci$ ($0.29 \mu g$) of L-[U- ^{14}C]proline. Cell walls were then prepared and hydrolysed, and the amino acid fraction was chromatographed (see above). (f) Protein was recovered from the sodium dodecyl sulphate-soluble fraction of the [^{14}C]tyrosine-labelled cells (b, above) by precipitation with 15% (w/v) trichloroacetic acid at $10^\circ C$ for 16 h, washed with more 15% trichloroacetic acid, dried *in vacuo*, and analysed as described for the cell walls.

Fig. 3. *Partial dinitrophenylation of amino acids*

The Figure shows an electrophoretogram of amino acids partially dinitrophenylated with fluorodinitrobenzene. ⦿, yellow spots; ○, ninhydrin-positive spots. The tracks are: (a)–(d) isodityrosine treated with fluorodinitrobenzene for (a) 0.1 min, (b) 1 min, (c) 5 min and (d) 25 min; (e) dityrosine treated for 5 min; (f) lysine treated for 5 min; (g) tyrosine treated for 5 min; (h) alanine treated for 5 min; (i) aspartic acid treated for 5 min; (j) markers (ethidium bromide and picric acid). According to the interpretation given in the text, the spots are (Dnp, 2,4-dinitrophenyl): (1) isodityrosine; (2) *O*-Dnp-isodityrosine; (3) *N*-Dnp-isodityrosines plus *NO*-diDnp-isodityrosines; (4) *NN'*-diDnp-isodityrosine plus *NN'*-*O*-triDnp-isodityrosine; (5) dityrosine, *O*-Dnp-dityrosine and *OO'*-diDnp-dityrosine; (6) *N*-Dnp-dityrosine etc.; (7) *NN'*-diDnp-dityrosine etc.; (8) lysine; (9) *N*-Dnp-lysines; (10) *NN'*-diDnp-lysine; (11) tyrosine; (12) *O*-Dnp-tyrosine; (13) *N*-Dnp-tyrosine plus *NO*-diDnp-tyrosine; (14) alanine; (15) *N*-Dnp-alanine; (16) aspartic acid; (17) *N*-Dnp-aspartic acid.

wide range of soluble proteins *in vitro* (LaBella *et al.*, 1968; Aeschbach *et al.*, 1976) by use of H_2O_2 plus plant peroxidase. There is evidence that extracellular peroxidase is also responsible *in vivo* in sea-urchin egg vitelline membranes (Foerder & Shapiro, 1977). It is not clear why plant cell-wall glycoprotein should form isodityrosine (an ether) instead of dityrosine (a biphenyl). However, its biosynthesis is very probably analogous to lignin synthesis, which occurs in the cell wall via peroxidase-generated free radicals and results in a mixture of ether and biphenyl bonds (for review see Harkin, 1967).

A reaction catalysed by extracellular peroxidase should be inhibited by the strong reducing agent dithiothreitol. Sub-lethal doses of dithiothreitol suppressed the formation of isodityrosine by potato cells, and also lowered the proportion of newly synthesized protein that became covalently bound in the cell wall (Fig. 5). Cooper & Varner (1981) have also claimed that inhibition of peroxidase or withdrawal of H_2O_2 (methods not given) suppresses wall–protein binding. The results (Fig. 5) support the

view that isodityrosine formation is responsible for wall–protein binding. Dithiothreitol also suppressed hydroxyproline formation (results not shown), but it has been established by use of a more specific inhibitor of prolyl hydroxylase (2,2'-bipyridyl) that the hydroxylation of proline is not necessary for wall–protein binding (Holleman, 1967; Smith, 1981; cf. Whitmore, 1978). The effect of dithiothreitol on isodityrosine formation was more pronounced than that on wall–protein binding: this is to be expected if several isodityrosine bridges are formed to each polypeptide molecule, as suggested above.

Possible consequences of glycoprotein cross-linking

Two speculations seem admissible: (1) the cross-linking of wall glycoprotein molecules through isodityrosine bridges might rigidify the wall and suppress cell growth (Lampert, 1970; Fry, 1979); (2) the degree of cross-linking might be limited by the wall peroxidase concentration (Fry, 1979; Lampert, 1978), which is under hormonal control (Fry, 1980). The first contention is supported by the finding that treatment with dithiothreitol

Fig. 4. Action of acidified sodium chlorite on isodityrosine
Purified isodityrosine ($10\mu\text{g}$) was incubated at 57°C in $20\mu\text{l}$ of 0.3% NaClO_2 containing 0.12% acetic acid. The reaction was stopped after a specified time by the addition of $2.5\mu\text{l}$ of 1M-NH_3 . The products were separated by t.l.c. on silica gel in solvent 5, and stained with a spray of Folin–Ciocalteu phenol reagent followed by NH_3 vapour. Track 1, chlorite-free control; tracks 2–8, treated with chlorite for 0.2, 2, 5, 10, 15, 30 and 105 min respectively. (Similar results were also obtained if the plate was stained with ninhydrin.)

loosens the cell wall (Thompson & Preston, 1968); the second is supported by the fact that the formation of isodityrosine never goes to completion, so that some unmodified tyrosine residues always remain in wall glycoprotein (Lamport, 1977). Taking (1) and (2) together leads to the idea that the long-term growth-promoting action of certain plant hormones (e.g. gibberellins; Fry & Street, 1980) may be mediated by a suppression of peroxidase secretion.

The structure of isodityrosine is, like dityrosine, well-suited to the permanent cross-linking of structural proteins. Its discovery strengthens the general view (Fry, 1982) that polymer-bound phenols play a crucial role in determining the nature of the primary cell wall in higher plants.

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Fig. 5. Effect of dithiothreitol on isodityrosine synthesis and wall-protein binding

Samples (5 ml) of an exponentially growing potato cell-suspension culture were incubated for 13 h with $0.3\mu\text{Ci}$ of $\text{L-[U-}^{14}\text{C]tyrosine}$ and 0mM -, 0.4mM -, 2mM -, 10mM - or 50mM -dithiothreitol. After this, 5 ml of 6% (w/v) sodium dodecyl sulphate containing 2% (v/v) 2-mercaptoethanol and 2% (w/v) NH_4HCO_3 was added, and the method described for the preparation and hydrolysis of cell walls was followed. In addition, the sodium dodecyl sulphate-soluble protein was precipitated with trichloroacetic acid (final concentration 12.5% , w/v; 4°C for 1 h), washed with more trichloroacetic acid, freeze-dried and hydrolysed. The amino acids from the hydrolysates were examined for $[^{14}\text{C}]$ tyrosine and $[^{14}\text{C}]$ -isodityrosine by the method indicated for Fig. 2. From these results were calculated the following: ○, $[^{14}\text{C}]$ isodityrosine as a percentage of the total radioactivity in protein; ●, wall-bound $[^{14}\text{C}]$ protein as a percentage of total $[^{14}\text{C}]$ protein. The absolute values for incorporation of radioactivity into total protein (▽) are also shown. Similar results were obtained in two independent repeats of this experiment, including one with a culture of 'Paul's Scarlet' rose (*Rosa* sp.).

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