BIOSYNTHESIS OF CELL WALL PROTEIN: SEQUENTIAL HYDROXYLATION OF PROLINE, GLYCOSYLATION OF HYDROXYPROLINE AND SECRETION OF THE GLYCOPROTEIN*

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Received April 13, 1970

SUMMARY: The biosynthesis of the hydroxyproline-rich cell wall protein involves several discrete cytoplasmic steps: the assembly of the polypeptide chain; the modification of this polypeptide through the hydroxylation of proline residues; the glycosylation of most of these hydroxyproline residues with arabinose. The glycoprotein is then transported to the cell wall.

The primary wall of the plant cell contains a glycoprotein, the protein moiety of which is rich (up to 25% of the residues) in hydroxyproline (8). Recently, Lamport (9,10) demonstrated that most of these hypro** residues are glycosylated with arabinose. Peptidyl-hypro is formed by the hydroxylation of peptidyl-proline (4). This reaction is carried out by a hydroxylating enzyme in the cytoplasm (5). The hydroxylated polypeptide is transported from the cytoplasm to the cell wall (2,3,13), a process which is mediated by subcellular organelles (3). In view of the fact that most of the hypro in the cell wall is glycosylated, we investigated the time sequence for the hydroxylation of peptidyl proline and the glycosylation of hypro and its relation to the transport of this protein to the cell wall.

When thin disks of carrot phloem parenchyma tissue are incubated, the synthesis of the hypro-rich cell wall glycoprotein is induced (3). In these experiments we used tissue disks (1 mm in thickness and 7 mm in diameter) which were preincubated for 24 hours at 30° in water containing 50 µg/ml of chloramphenicol to inhibit bacterial growth (11). Such disks normally

*Supported by the U.S. A.E.C. contract AT(04-3)-34, Project 159-2.

**Abbreviations used: Hydroxyproline = hypro.

synthesize the hydroxyproline-rich protein at a constant rate and vigorously incorporate radioactive proline.

Carrot disks were labeled with ^{14}C-proline as described in Fig. 1, rinsed with 1 mM proline at 4 C, and immediately homogenized with a mortar and pestle in 0.3 M sucrose containing 0.01 M phosphate buffer pH 6.0, also at 4 C. Three aliquots of this homogenate were precipitated separately with an equal volume of 15% trichloro-acetic acid and the proteins were collected on membrane filters. Total incorporation was determined on one set of filters by liquid scintillation counting. Another set of filters was sealed in vials each with 5 ml of 6 N HCl and autoclaved for 90 minutes at 120 C and 22 pounds pressure (control experiments showed that this gave the same result as hydrolysis at 110 C for 18 hours). The hydrolysates were concentrated by evaporation under vacuum, and chromatographed on Whatman 3 MM chromatography paper for 16 hours in isopropyl alcohol:formic acid:water, 15:2:2, to separate proline from hypro (3). The papers were cut into 1 inch pieces and the radioactivity in each piece was determined. This allowed us to calculate first the percentage of the radioactivity in the chromatographed protein hydrolysate which is in hypro and then the total amount of ^{14}C-hypro per gram of tissue.

The third set of filters was subjected to alkaline hydrolysis in order to break the peptide bonds but preserve the glycosidic bonds. Hydrolysis was carried out in sealed vials at 120 C for 15 hours in 0.43 M $Ba(OH)_2$ (9). The hydrolysates were neutralized with sulfuric acid, centrifuged, concentrated, and chromatographed as above. When sufficient material was used, application of Ehrlich's reagent indicated two hydroxyproline positive spots: one in the location of free hydroxyproline (about 5 inches from the origin, under our conditions where the solvent runs off the paper) and one about ½ inch from the origin. The latter spot contained the hydroxyproline glycosides, the identity of which is discussed below. The chromatograms were cut into ½ inch pieces and the radioactivity determined in each piece. This

allowed us to calculate the percentage of radioactivity in the alkaline hydrolysate which was in glycosylated hypro and then the total amount of glycosylated ^{14}C-hypro per gram of tissue. Alkaline hydrolysis of the protein always yielded some free hypro presumed to be unglycosylated in the protein. The total amount of hypro found was the same, using either method of hydrolysis.

When carrot disks were labeled with ^{14}C-proline the amino acid became incorporated at a linear rate after a 3 minute lag-period (Fig. 1). The lag varied from 1 minute to 5 minutes depending on the tissue. Initially the proteins contained little ^{14}C-hypro, but after some time, it also increased linearly; after 25 minutes approximately 20% of the total radioactivity could be accounted for as hypro. About 10 or 12½ minutes after the addition of the label there was a considerable amount of ^{14}C-hypro in the

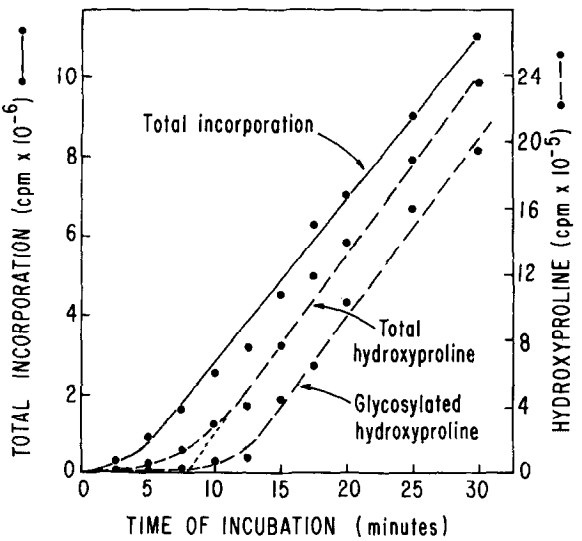

Fig. 1: Time course of incorporation of ^{14}C-proline into proteins and synthesis of ^{14}C-peptidyl-hydroxyproline and ^{14}C-peptidyl hydroxyproline-glycoside. One gram lots of carrot disks were incubated with 2 µc of ^{14}C-proline (New England Nuclear, spec. act. 1 µc/4 mµmoles) in 10 ml of 2 mM phosphate buffer pH 6.0 with 50 µg/ml of chloramphenicol. The data were obtained by using aliquots (1/10) of the entire homogenate to determine total incorporation, percent of total counts in hydroxyproline and percent of total counts in hydroxyproline-glycoside, and multiplying the numbers obtained by the appropriate factors so that all numbers on the graph indicate cpm/gram of tissue.

proteins but very little of it was glycosylated. Subsequently, radioactivity in glycosylated hypro increased linearly. Extrapolation of the slopes suggests that glycosylation lags about 4 minutes behind hydroxylation which in turn lags about 4 minutes behind incorporation. The data show that after 20 minutes most (85%) of the hypro residues were glycosylated.

In a separate experiment we examined the appearance of ^{14}C-hypro in the cell wall proteins. Disks were incubated with ^{14}C-proline as shown in Fig. 2 and homogenized in water. Total incorporation was determined as in the first experiment. The cell walls were isolated by centrifugation at 1000 x g for 3 minutes and rinsed 3 times with cold water. Aliquots of a cell wall suspension were collected on membrane filters, washed, and subjected to acid or alkaline hydrolysis. The incorporation of ^{14}C-proline into the cellular proteins proceeded linearly without appreciable lag, but the appearance of ^{14}C-hypro in the cell wall proteins had a lag of 15 minutes (see 3). Most (85%) of this hypro was glycosylated at all times and there was no lag between the arrival of free and glycosylated hypro in the cell wall suggesting that the hypro residues were already glycosylated when the protein was secreted.

Further experiments demonstrated that in carrot phloem parenchyma tissue as in other tissues (Lamport, personal communication) hypro is glycosylated with arabinose. When the neutralized basic hydrolysate was chromatographed on a cation exchange resin (10), it was possible to purify the hypro glycosides. Acid hydrolysis of these glycosides followed by chromatography showed that the sugar attached to the hypro co-chromatographed with arabinose. When these hypro arabinosides were chromatographed on paper (as discussed above) they formed a single spot about ½ inch from the origin and gave a positive color reaction with Ehrlich's reagent.

These kinetics of the incorporation of proline, the formation of peptidyl-hypro and its subsequent glycosylation suggest that the appearance of the hypro-rich glycoprotein in the cell wall is preceded by several

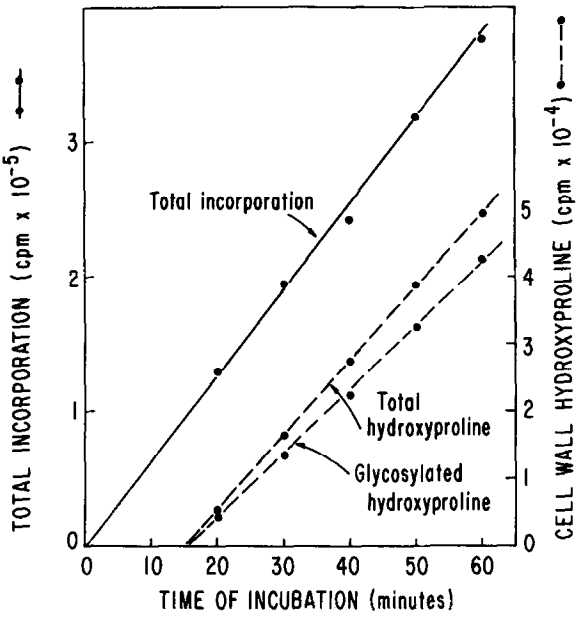

Fig. 2: Time course of incorporation of ^{14}C-proline into proteins and appearance of ^{14}C-peptidyl-hydroxyproline and ^{14}C-peptidyl hydroxyproline-glycoside in the cell wall fraction. One gram lots of carrot disks were incubated with 1 μc of ^{14}C-proline and 0.2 μmoles of proline in 10 ml of 2 mM phosphate buffer pH 6.0 with 50 μg/ml of chloramphenicol. The data were obtained by using aliquots (1/10) of the entire homogenate or of a cell wall suspension to determine total incorporation or percent of counts in the cell wall in hydroxyproline or hydroxyproline glycoside, and multiplying the numbers obtained by the appropriate factors so that all numbers on the graph indicate cpm/gram of tissue.

distinct cytoplasmic events: (1) The assembly of the polypeptide chain; (2) the modification of this polypeptide chain through hydroxylation of certain proline residues; (3) the glycosylation of most of the hypro residues; and (4) the transfer of the glycoprotein from the cytoplasm to the cell wall. In collagen synthesis, the hydroxylation of lysine and the glycosylation of hydroxylysine are also sequential cytoplasmic steps (15).

The hypro-rich molecules which are on their way to the cell wall are at all times associated with membranous organelles in the cytoplasm (3). Evidence obtained with several mamalian systems indicates the glycosylases involved in glycoprotein synthesis are associated with membranous organelles (see for example 1,6,7,12). There is as yet no information concerning

the subcellular localization of glycosyl transfer enzymes involved in glycoprotein synthesis in plant cells. Ray et al. (14) recently demonstrated that a glycosyl transferase involved in the synthesis of cell wall carbohydrate (cellulose) is associated with the golgi apparatus. Collectively, these observations suggest that glycosyl transfer enzymes involved in glycoprotein biosynthesis in plant cells may also be associated with the membranous organelles.

I thank Mrs. Marcia Doerschug and Mrs. Rebecca Roberts for their help with these experiments.

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