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HYDROXYPROLINE-CONTAINING PLANT PROTEINS

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Data on hydroxyproline-containing proteins of plant cell walls were reviewed. Structure—function relationships were discussed.

Key words: extensin, extensin-like proteins, arabinogalactan proteins.

Extensin is the principal hydroxyproline-containing glycoproteide of the plant primary cell wall. This protein was first isolated by Lamport and Northcote, who named it extensin and showed that it is the most important structural component of the primary cell wall and imparts strength and flexibility to it [1]. Then thoughts about extensin-like proteins (ELP) changed. It has been thought that proteins perform activities important to cell and organism metabolism, which explains the increased interest in them [2-11]. Thus, an increase in the extensin content in the cell wall caused the cell to become resistant to lysis in hypotonic medium and pathogenic infection [2, 3], inhibited further growth elongation [5, 6], preserved a given cell morphology [7], and elicited protective functions during stressful situations, like lectins. The extensin content increased sharply in cell culture, upon damage from wounds and infection, and under the influence of low temperature and ethylene [2, 12-16]. Carrot extensins are coded by a small multigene family. Various transcripts are activated in response to various signals. Wounding leads to the appearance of transcripts of 1.5 and 1.8 thousand base pairs that are coded by a single gene but have different transcription starting points. However, ethylene treatment activates transcripts of 1.8 and 4.0 thousand base pairs. The properties of extensin promoter have been studied [17, 18]. It was shown that the level of GUS (β-glucuronidase) expression from extensin promoter was several times higher in callus than in organs of transgenic carrot, i.e., carrot extensin promoter worked effectively in nondifferentiated carrot callus cells. It is assumed that selective activation of such genes is the mechanism for accurate morphogenetic control over the assembly of the cell wall during cell differentiation.

Cell-wall proteins participate in interactions between cells involved in recognition processes [19-22] and also carry out various functional activities [23-25].

Plants are known to contain at least three classes of hydroxyproline-rich glycoproteides: certain lectin-like proteins found in Solanaceae [26], arabinogalactan proteins (AGP) [27-29], and extensins [1, 13, 30, 31].

The first group includes hemagglutinating glycoproteides whose activity is inhibited specifically by di- and tri-N-acetylglucosamines [26]. Potato afforded a protein that was localized in the cell wall and had hemagglutinating activity. The protein content increased sharply upon wounding the plant, which indicates that this protein is involved in the protection strategy [32]. A hydroxyproline-containing protein with hemagglutinating activity was isolated from *Datura innoxia*. The carbohydrate part of lectin did not exhibit binding activity *in vitro* [33, 34]. Lectin isolated from *Datura innoxia* in cell culture of CML derived from murine melanoma B-16 showed high proliferative activity. The protein was found to be a mitogen [35].

AGP are localized primarily in the intercellular matrix and occur in either the free state or associated with the plasmatic membrane. They are the main component of plant gum and exudate. AGP also accumulate upon wounding of plants, like for other hydroxyproline-rich proteins (HRGP) [27]. Interesting results were obtained from a study of proteins in bean root nodules [22]. Thus, HRGP accumulation in bean-nodule coating was regulated during nodule formation. The highest protein content was observed 10 weeks after innoculation. Hydroxyproline (Hyp) accumulated in the nodule coating and pith during nodule ripening. The coating had about 60% Hyp in all analyzed development stages. About 80% of Hyp in the nodule skin was localized in the cell wall. Like extensin, it was solubilized from the cell wall by $CaCl_2$ (0.2 M). These extensin-like proteins participate in formation of the protective function in nodules, like extensin isolated from bean seed covering [36]. On the other

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hand, about 60% of Hyp in the pith, the zone that contains nitrogen-fixing bacteria, occurred in the AGP. In general, the AGP content in the bean nodule pith was much greater than in the coating, whole nodule, roots, leaves, flowers, pods, and seeds [22]. It is assumed that AGP participates in intercellular recognition and is involved in plant interaction with the bacterial cell or with the support of this interaction during nodule development.

Extensins are structural glycoproteides that have a very complicated structure and, as a result, a polyfunctional type of activity [37-39]. ELP isolated from bean, carrot, potato, tobacco callus, tomato, etc. have been studied in most detail [8, 13, 30, 36, 39, 40, 41]. Extensins typically have high contents of Hyp and basic amino acids (Tyr, Val, Ser, Lys). Carbohydrates make up about two thirds of the glycoproteide mass.

Two families of HRGP ($HRGP_1$ and $HRGP_2$) were isolated from melon callus. $HRGP_1$ was identified as an AGP. The carbohydrate content was about 94%, mainly galactose (66%) and arabinose (34%). Polysaccharide chains of carbohydrates were bound to Hyp. Several fractions were obtained from $HRGP_2$. The main components, $HRGP_{2a}$ and $HRGP_{2b}$, contained 41% protein and 50% carbohydrate. Biochemical and immunochemical methods determined that $HRGP_{2b}$ may be a precursor of cell-wall HRGP [42].

A galactose-rich principal glycoproteide (GBGP) from tobacco cell culture was purified and characterized. GBGP and extensin made up a large part of the cell-wall glycoproteides. The carbohydrate composition of GBGP consisted mainly of galactose (72%) with arabinose (17%) and mannose (7%) also present. Immunochemical methods showed that the protein was localized. Thus, GBGP was specific to the parenchyma whereas extensin was localized in the epidermis. Such tissue specificity and distribution features indicate that these basic proteins play a significant functional role [43].

The accumulation and composition of HRGP during soy seed ripening was studied. It was found that extensin appeared in cell extracts on the 16-18th day and reached the highest concentration on the 26th day. The outer layer of the seed coating was especially rich in this protein compared with the roots, leaves, stems, and flowers. The content of basic amino acids such as histidine and lysine was relatively high whereas that of acidic amino acids was low. This means that the protein is basic and that this property is important for interacting with other structural components, in particular, cell-wall polyuronates. The protein contained mainly arabinose with a small amount of galactose bound to serine [36]. The carbohydrate and amino-acid compositions were very similar to those described previously for Hyp-containing glycoproteides isolated from carrot [44], potato [40], tobacco callus [41], and tomato suspension culture [13]. It was found that protein was concentrated mainly in cell walls of palisade cells and in cytoplasmic and cell walls of "hourglass" cells, which is consistent with a tissue-specific protein [45].

A glycoproteide called potato agglutinin was isolated from potato grown in a greenhouse at 21°C with a 14-h light period [40]. Its amino-acid composition was reminiscent of the cell-wall glycoproteide extensin [1] and the Hyp-rich protein isolated from carrot [8]. These proteins were rich in Hyp, Ser, and Lys and contained smaller quantities of Met, Cys, Phe, and Arg [1, 8, 46]. The carbohydrates contained mainly arabinose but also had minor amounts of galactose and glucose [38]. On the other hand, the structure of potato agglutinin was similar to that of potato lectin [26]. However, significant differences were observed in the biological activity of potato lectin [26] and agglutinin [40]. Thus, potato agglutinin agglutinated more strongly the avirulent strain *Pseudomonas solanacearum* although the hemagglutinating activity was weaker. In contrast with this, potato lectin agglutinated more strongly erythrocytes but did not agglutinate bacterial cells. The hemagglutinating activity of potato lectin increased in subsequent purification stages whereas the agglutinating activity for bacterial cells decreased. Potato agglutinin was localized in the cell wall and precipitated bacterial lipopolysaccharides [47]. Therefore, the proteins may play a significant role in binding bacteria to the cell.

HRGP isolated from potato suspension culture [48] had amino-acid and immunoenzyme analyses and a carbohydrate composition that were very similar to tomato extensin [30] rather than to potato agglutinin [40] and lectin [26].

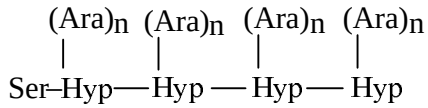
An analysis of the literature indicates that ELP from the Solanaceae and Fabaceae families are most studied. Cotton ELP are practically unstudied. This is explained most probably by the exceedingly low solubility of these glycoproteides. These proteins may be more strongly bound to the cell wall or irreversibly bound in part. Nevertheless, research in this area is underway. Thus, extensin or extensin fragments were isolated and characterized by electrophoresis from cell walls of cotton (*Gossypium hirsutum* L.) suspension culture using various selective hydrolysis methods with subsequent gel filtration [49]. The three most frequently repeating sequences were identified: a) Ser-$(Hyp)_6$-Ser-Hyp-Hyp-Lys, b) Ser-$(Hyp)_4$-Val-Lys, c) Ser-Hyp-Hyp-Ser-Ala-Hyp-Lys. It is assumed that the exceedingly low solubility of cotton extensin was related to protein—protein or protein—phenol—protein in addition to pectin—protein interactions [49].

Hyp-containing ELP were isolated by the Lamport method [30] from 2-day sprouts and suspension culture of cotton [50-52]. Hyp was determined using phenylthiocarbamoyl (PTC) derivatives [50]. The protein:carbohydrate ratio was measured

for ELP isolated from suspension culture (1:3) and 2-day sprouts (20% carbohydrate) of cotton. Paper chromatography detected arabinose and minor amounts of galactose. Electrophoresis revealed two polypeptide bands of glycoproteide nature [52]. The proteins did not exhibit hemagglutinating activity. However, cell aggregation was observed in CML cell culture at a protein dose of 100 μg/mL [53]. The exceedingly low solubility and corresponding low yield of protein should be mentioned. This is evidently related, on the one hand, to protein—phenol (gossypol) or protein—pectin interactions [49] and, on the other, to inter- and intramolecular *iso*-dityrosine (IDT) linkages [54].

The principal differences in extensins from various cell types (tomato, tobacco, carrot, etc.) are the periodicity of the polypeptide sequences and the distributions of tyrosine linkages and glycosylated fragments.

Two groups of soluble extensins (P-1 and P-2) that are precursors of the insoluble extensin incorporated into the cell-wall matrix were observed [30]. These polypeptides were rich in Ser, Tyr, Lys, and glycosylated Hyp. The carbohydrates were bound to Hyp through O-glycoside bonds [55-58]. The primary structure of the protein polypeptide chain isolated from tomato suspension culture was highly periodic and consisted for P-1 of the deca- and hexadecapeptides Ser-$(Hyp)_4$-Thr-Hyp-Val-Tyr-Lys and Ser-$(Hyp)_4$-Val-Lys-Pro-Tyr-His-Pro-Val-Tyr-Lys. P-2, in contrast with P-1, was formed by sequences of the di- and octapeptides Tyr-Lys and Ser-$(Hyp)_4$-Val-Tyr-Lys and a small amount of similar decapeptides bonded by interpeptide IDT linkages Ser-$(Hyp)_4$-Val-1/2 IDT-Lys-1/2 IDT-Lys [30]. Hyp-containing glycoproteides typically contained a repeating sequence of the pentapeptide Se-$(Hyp)_4$ although in certain instances repeating sequences containing five or six Hyp were observed [49, 59]. Glycans incorporated into ELP consisted mainly of arabinose and galactose. Arabinose was bound to Hyp whereas single galactose units were bound to Ser [30, 60, 61]. The interactions of Hyp and arabinose are illustrated below.



where n = 1-4 arabinose units.

The repeating sequences without carbohydrates were established using polyclonal antibodies against glycosylated and unglycosylated epitopes of the soluble precursors P_1 and P_2 [62]. It is assumed that these sequences participate in inter- and intramolecular cross-linkages in extensin and possibly IDT linkages for creating the network structure [63].

The repeating sequence $(Hyp)_4$ with varying amounts of arabinose is evidently biologically important because it carries a definite functional load. Physicochemical methods showed that carbohydrates bound to $(Hyp)_4$ supported the extensin molecule in the elongated polyproline II conformation [44, 64, 65]. A molecular model of the protein has demonstrated that the *tetra*-arabinoside can form intramolecular H-bonds and stabilize this conformation [64, 66]. Molecular modeling has determined that the $(Hyp)_4$ sequence has a random twisted conformation. However, a second order β-spiral forms upon binding to arabinose. On the other hand, three arabinose units bound to one Hyp are twisted to form a cavity (0.5 × 0.6 nm) stabilized by intramolecular H-bonds. The H-bond between Ara—Ara is unstable and exists in only one of the possible transition states, the energy parameters of which are similar [67]. Evidently such a structure imparts rigidity to the molecule whereas the $(Hyp)_4$ sequence fulfills a structure-supporting role. If one arabinose unit is bound to $(Hyp)_4$, a clearly discernable cavity is not observed and the second order β-spiral is retained. As a result, addition of the next amino acids (Val, Lys, IDT-isodityrosine) forms a three-dimensional comb-like structure. Oligosaccharide functional groups involved in recognition processes and then in carbohydrate—carbohydrate and carbohydrate—protein interactions may become more available. Introducing IDT units causes internal structural rearrangements due to local changes of torsion angles. This changes the energy contribution in the nonpolar part of the fragments. The structure as a whole becomes energetically favorable. The IDT units are located in the frontier region of the fragments. Such a structure may guarantee a sterically favorable conformation for participation of the molecules in inter- and intramolecular interactions [51, 67].

The new phenolic amino acid IDT was observed in plant proteins of the cell wall only. It is the product of post-translation biosynthesis effected by peroxidase-catalyzed linking of two tyrosine units [54]. It was demonstrated that extensin was secreted into the cell wall as a monomer and then covalently bound to other molecules through IDT [54] or di-IDT linkages [63]. Thus, IDT forms inter- and intramolecular cross-linkages. The extensin monomer formed a dimer, trimer, and oligomer. The cell wall matrix became rigid [68]. Evidently this was responsible for their exceedingly low solubility [69, 70], which depended on the peroxidase concentration and was controlled by hormones, in particular, gibberellin [71-74]. Inhibition of extensin peroxidase activity decreased the cell-wall rigidity, elongated the cells, and caused more vigorous growth. Thus, an

inhibitor that inhibited cross-linking of extensin *in vitro* was isolated from cell walls of tomato suspension culture. It was assumed that the inhibition of tomato hypocotyl growth was related to the accumulation of extensin in cell walls and was regulated by extensin peroxidase of molecular weight 58.5 kDa [75].

An analysis of the literature indicates that inter- and intramolecular linkages have a more complicated structure. Thus, tyrosine trimer, called pulcherosin, was isolated from the hydrolysate of primary cell walls from tomato suspension culture. It was found that the molecule consisted of IDT and tyrosine units. It is assumed that the three tyrosine units in pulcherosin cannot be situated next to each other because of steric hindrance. Therefore, they form interpolypeptide cross-linkages or loops inside the polypeptide chain [68, 76].

Research on the structural features of ELP are directed toward obtaining cDNA that codes the sequences of this protein. It has been shown that the polypeptide contains repeating Ser-$(Pro)_4$ sequences that are typical of Hyp-containing proteins and frequently encountered Val—Tyr—Lys sequences [77-80]. Thus, cDNA (clone 6PExt 1.2) that codes the extensin sequences was obtained from 6-hour mesophilic tobacco (*Nicotiana sylvestris*) protoplasts. Sequences characteristic of HRGP such as the repeating Ser—$(Pro)_4$ sequence were established for the protein. The possibility that intermolecular IDT linkages could form was emphasized by the presence of 4-unit Tyr—X—Tyr—Lys; intramolecular linkages, by the Val—Tyr—Lys sequence [79]. The only copy of the extensin gene (atExt1) was obtained from *Arabidopsis thaliana* L. Heynh. The protein contained 374 amino acids and the highly repetitive Ser—$(Pro)_4$ and Ser—$(Pro)_3$ sequences. Tyr—X—Tyr—Lys (2 copies) and Val—Tyr—Lys (13 copies) were also observed. It is assumed that namely these sequences are responsible for formation of inter- and intramolecular linkages [80].

Mainly tri- and tetra-arabinolyzed Hyp units occurred in unified [81] and growing [35, 82] cells. Thus, cell walls of melon infected with anthracnose from *Colletotrichum logenarium* became sensitive to glycosylation. Mainly tri- and tetra-arabinolyzed Hyp units were found [81]. Formation of the arabinoside may be one of the mechanisms for protecting cells from infection. This probably occurs because molecular modeling showed that the fourth Ara on Hyp causes conformational rearrangements. The last Ara seems to shield the cavity and is situated in a different plane [67, 83].

Researchers are studying the localization of extensin and its assembly and interaction with other cellular components because this information gives an indication of the involvement of the extracellular milieu in growth, development, and plant-cell functions. The use of monoclonal antibodies (MAb) to various epitopes of ELP provided a large amount of information on this [84-87]. Thus, the MAb LM1 with a high affinity for the Hyp-rich protein determinant helped to show that the antibody bound to hydrophobic proteins of the plasmatic membrane and rice protoplasts. On the other hand, the LM1-epitope interacted with four glycoproteides localized in the upper part of rice roots. It was assumed that the epitope localized on the cell surface owing to the development of protoxylem and metaxylem in the stele, elongation of the walls in epidermal cells, and formation of capsule-like structures in the upper part of the roots [84].

One of the MAbs (11.D2) to the cell membrane of tobacco suspension culture was also specific to tomato extensin but not to potato lectin and arabinogalactan proteins [85]. It showed no affinity to protoplasts quickly obtained from leaves. However, incubation of these protoplasts in the appropriate medium helped to reveal the epitope with affinity for the MAb 11.D2.

Interaction of plants with a pathogen was studied by producing MAbs that recognized glycoproteides of the extracellular matrix [86-89]. It was demonstrated that MAbs LM11, LM12, and LM20 recognized epitopes of HRGP [87]; MAC204 and MAC265, epitopes of glycoproteides obtained after infection [88]. Two groups of newly synthesized proteins were found in response to infection of cherries (*Brassica*) by the avirulent strain *X. campestris*. It was demonstrated that MAbs LM20 and MAC204 recognized epitopes gp160; LM11, LM20, and MAC204; gpS. These proteins were established as ELP that appear in response to infection of a plant by a pathogen.

Hybridomas that produce MAbs to cotton membrane proteins isolated from 2-day sprouts have been produced. Immunochemical analysis showed reactivity of MAb-2C82 and 3A10 with isolated membrane proteins (2-day sprouts), ELP (cotton suspension culture), protoplasts (cotton callus), and lectin-like proteins (cotton seeds) [90-92]. Based on the results, it has been proposed that common binding regions exist for ELP, membrane proteins, and protoplasts. These results have indicated that plant ELP are not only structural proteins but also complicated polyfunctional agents. The polyfunctionality of the protein may be related to structural features, in particular, the variability in the steric structure of the oligosaccharides. It was shown [32] that STA protein (*Solanum tuberosum* agglutinin) was bound to the cell wall by H-bonds between the glycosylated region and the polysaccharide matrix of the cell wall. The binding site, which was located in the nonglycosylated region, evidently carried a definite functional load on the external cell surface. The proposed model [32] was similar to the

model of membrane proteins with a hydrophobic domain immersed in the membrane with a hydrophilic region exposed on the outer cell surface [93].

On the other hand, the polyfunctionality of the protein might also be due to the more complicated structure of the ELP [23, 24, 28, 59, 93-95]. Thus, the gene product Pexl (pollen-extensin-like), which is specific to corn pollen, has been identified. It contained the often repeated sequence Ser-$(Pro)_n$. It was observed with extensin bound to the cell wall. Analysis of the sequence indicated that the protein had a global domain at the N-terminus and an extensin-like domain at the C-terminus. Pexl was expressed exclusively in pollen and was not observed in vegetative and female organs nor induced in leaves after wounding. It is assumed that these proteins can participate in reproduction or act as structural elements of the wall of pollen tubes during their rapid growth or as molecules determining the sex and interacting with the partner molecules in the pistil [23].

Such polyfunctionality of ELP is very important in studying the molecular bases upon which cell-response systems are functionalized by the action of external factors in the plant environment. This causes researchers to study all aspects of these proteins. One of the approaches is the biosynthesis of the most important cell biopolymers, nucleic acids and proteins [96].

The biological activity of cotton ELP was studied in tests of plant and animal cells. The biological activity was evaluated by both the level of synthesis of nucleic acids using incorporation of ^{3}H-thymidine and its effect on protein biosynthesis in isolated cotton nuclei *in vitro* using incorporation of ^{32}S-methionine [51-53, 97, 98]. It was demonstrated that ELP affected various cell types and isolated nuclei by suppressing cell proliferation. The observed differences obviously reflect the nature of the interaction of these proteins with the cell surface (cotton suspension culture, myeloma cell K-562). Clearly this is due to structural factors in the carbohydrate fragments of glycoproteides in the ELP and in membrane receptors of target cells, the interaction of which provides, along with other factors, a mechanism for the direct antiproliferative response of the cells.

The effect of ELP on the intertwined stable CML cell culture has been studied in most detail. It was found that ELP caused cells to aggregate at a protein dose of 100 μg/mL. This was apparently due to the protective function of the cells [53]. Thus, the mechanism of action of ELP was studied on partially synchronized CML cell culture. The protein effect was determined using ^{3}H-thymidine incorporation and a living-cell census [53]. It was shown that the antiproliferative activity of ELP was related to the S-period of the cell cycle when transcription-active chromatin is most vulnerable to the action of external factors. It was found that cells perished (50%) at a protein dose of 100 μg/mL (cytotoxic effect) although DNA fragmentation was not observed [99]. It is assumed that ELP causes cells to perish through necrosis as a result of destruction of intracellular biochemical processes.

It was also shown that deglycosylated ELP were more actively cytotoxic, i.e., suppressed to a greater extent growth of murine melanoma cells than ELP themselves. Evidently this effect was due to structural features of the proteins, namely, the removal of oligosaccharide fragments unmasked the biological activity. Functional groups of oligosaccharides that participate in recognition processes probably then bound to target cells adopting a more favorable conformational (steric) condition. As a result, docking sites for the protein on the cell surface increased. This may explain the distinct antiproliferative action of deglycosylated ELP [51].

In summary, several common properies characteristic of ELP can be seen. These proteins contain Hyp. The tetrahydroxyproline (Hyp_4) sequence, arabinosylated to varying degrees, is repeated often in the protein structure. Arabinosylated Hyp_4 forms a second-order β-spiral, thereby supporting extensin in the elongated polyproline II conformation. IDT units observed in the protein evidently participate in the formation of inter- and intramolecular cross-linkages. It has been found that these proteins are involved in the plant protective strategy.

Thus, ELP have a very complicated structure and, as a result, a very complicated mechanism of action on cell functioning. Comparisons of the principal component of the animal cell matrix, collagen (a Hyp-containing protein), and plant ELP indicate that ELP are not only structural proteins involved in construction and elongation of cell walls but also complicated polyfunctional agents, i.e., components of many events occurring in cells such as differentiation, proliferation, and migration.

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