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STRUCTURE — FUNCTION RELATIONSHIP OF EXTENSIN-LIKE COTTON PROTEINS

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The structure-function relationship of extensin-like proteins (ELP) of cotton is studied by molecular modeling. The antiproliferative activity of deglycosylated ELP on a cell culture of KML murine melanoma is shown to be more expressed than the activity of the ELP themselves.

Key words: extensin-like proteins, cell culture, molecular modeling.

Structure—function relationships of glycoproteides (GP) are important to research on the mechanism of action and understanding of the functional principles of cellular regulatory systems [1, 2]. Extensin-like proteins (ELP), which possess a wide spectrum of physiological activity, are plant GP of great interest. They participate in the formation and extension of the primary cell wall and act as protectants during stress situations [3-5]. ELP isolated from plants of *Leguminosae, Solanaceae*, and other families have been studied in most detail to date [6-8]. Cotton ELP have practically not be studied.

We studied the effect of deglycosylation of cotton ELP in tissue culture in order to find the structure—function relationship.

ELP were isolated from 2-day cotton sprouts [9]. The quantitative content of total sugars, which was determined spectrophotometrically using anthrone—H_2SO_4, was 20%. Acid hydrolysis of ELP by 2 N H_2SO_4 with subsequent chromatography using butanol—pyridine—water revealed arabinose and trace quantities of galactose.

The presence of hydroxyproline, which is characteristic of extensin proteins, was determined using the phenylthiocarbamoyl (PTC) derivatives [10].

Cotton contains gossypol and its derivatives, which are highly toxic in tissue culture [11]. Therefore, the gossypol content of the isolated samples was studied using the qualitative reaction with phloroglucine. Gossypol and toxic phenolic compounds were not detected.

Cotton ELP were deglycosylated by anhydrous HF (see Experimental) under mild conditions that did not destroy peptide bonds [12]. The contents of protein and total sugars in the supernatant and precipitate were determined in order to confirm this. The supernatant, which contained the deglycosylated protein, contained ~5% of the total sugars. The precipitate contained no protein.

The biological activity of deglycosylated cotton ELP (d-ELP) was determined using the KML murine melanoma cell line during the logarithmic growth phase, a cytotoxic test for incorporation of ^{3}H-thymidine, and a count of live cells [13].

We have previously demonstrated that the effect of ELP on KML cells depends on the dose: 100 μg/ml suppressed incorporation of the label by 51% (CE_{50}); 50 μg/ml, by 27%; and 10 μg/ml, by 7%. The effect was analogous for counting live cells. Thus, a dose of 100 μg/ml suppressed cell growth by 48%; 50 μg/ml, by 23%; and 10 μg/ml, by 4%.

We selected a dose of 100 μg/ml, which was cytotoxic to 50% of the cells. About 80,000 cells were innoculated into 2 ml of nutrient medium (DMEM) with 10% bovine embryo serum, glutamine, and antibiotics in a vial. Substrate was added after one day. Cells were contacted with substrate for 24 h, after which ^{3}H-thymidine (10 μCi/vial, 1 mCi) was introduced for 1 h and live cells were counted. The results are given below:

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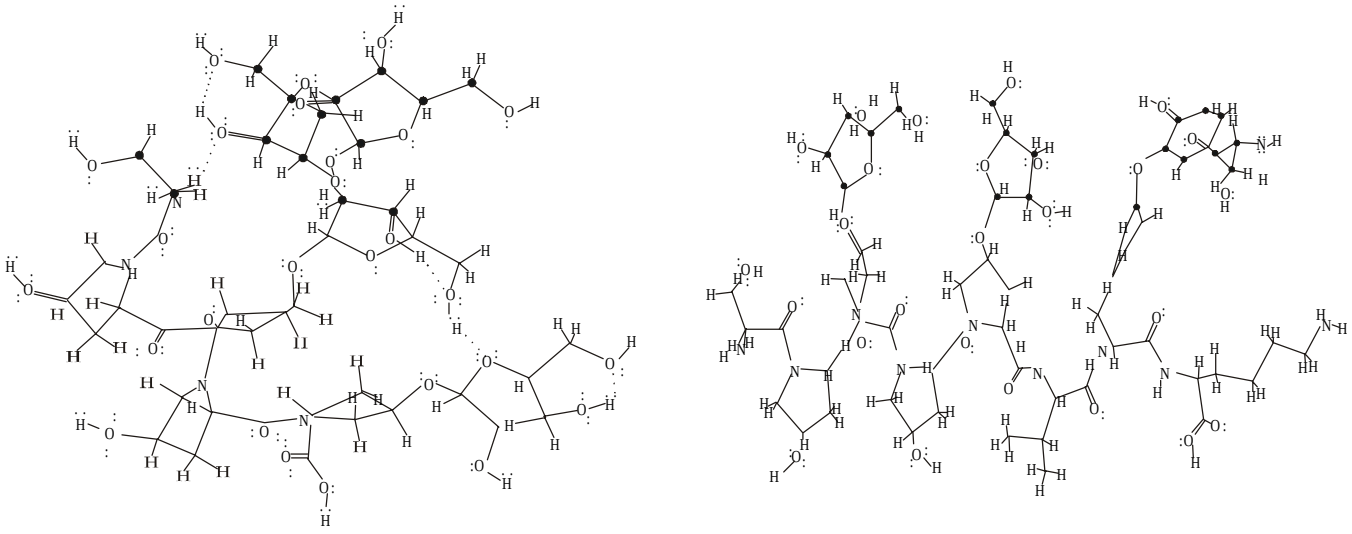
Sample	Suppression of ^{3}H-thymidine incorporation, %	Suppression of cell growth
Control	100	100
ELP	56	49
d-ELP	87	84

It can be seen that concentrations of 100 μg/ml (CE_{50}) suppress incorporation of ^{3}H-thymidine and cell growth by 56 and 49% for ELP and 87 and 84% for d-ELP in the two tests, respectively. The results indicate that d-ELP have a large cytotoxic effect, as hypothesized. An analogous effect is noted is laminin, the principal membrane-bound GP of animals, is used. Deglycosylated laminin and synthesized functionally significant fragments are more effective for delaying the growth of metastases [14, 15]. Therefore, the GP structure undergoes definite conformational rearrangements during deglycosylation. These change the biological activity.

A structure analysis of extensin isolated from various sources (tobacco, carrot, bean) revealed that the protein structure is highly conserved and contains often repeating sequences of tetrahydroxyproline arabinosylated to varying degrees (core-fragment) [6, 12, 16, 17]. These structural features are most probably extrapolated to the structure of cotton ELP.

A molecular model of the repeating core-fragment of extensin was constructed using the PCM-MMX program (Fig. 1, structure 1). Considering the capabilities of the program, we joined three Ara residues to the second Hyp; one Ara, to the fourth [18]. The structure was minimized for energy. Geometric and energy parameters in the ground state and with intramolecular H-bonds (arrows show the H-bonds) were calculated. The spatial structure shows that three Ara are twisted to form a cavity (0.5 × 0.6 nm) that is stabilized by intramolecular H-bonds (IMHB). IMHB form between serine and the third Ara and between the first Ara residues that are bonded to the second and fourth Hyp. The Ara—Ara H-bond is unstable and exists only in one of the possible transition states, the energy parameters of which are very similar.

The arabinosylated tetrahydroxyprolines form a second-order β-spiral. Obviously such a structure makes the molecule rigid and keeps the $(Hyp)_4$ in an extended conformation [19]. We also constructed a molecular model of one of the functionally significant fragments that was obtained by tryptic hydrolysis according to the literature (Fig. 1, structure II) [6]. The structure shows that whereas hydroxyproline is bonded to one Ara, a clearly defined cavity is not observed. The second-order β-spiral of $(Hyp)_4$ is retained.



I II

Fig. 1. Molecular models of extensin fragments:

I—Ser—Hyp—Hyp—Hyp—Hyp;
| |
$(Ara)_3$ Ara

II—Ser—Hyp—Hyp—Hyp—Hyp—Val—IDT—Lys.
| |
Ara Ara

With the addition of the last amino acids [Val, Lys, IDT (*iso*-dityrosine)], a comb-like structure forms and, possibly, functional groups of oligosaccharides that participate in recognition processes and subsequent carbohydrate—carbohydrate and carbohydrate—protein interactions, which become more accessible [20].

We determined that ~5% of the sugars become bound to proteins after deglycosylation. We propose that the sugar antennae, which recognize target cells and then bind to them, adopt a more favorable conformational (steric) state, as a result of which the number of attachment sites for protein on the cell surface increase. Apparently this explains the distinct antiproliferative activity of d-ELP.

Thus, d-ELP is demonstrated to be more cytotoxic, i.e., suppresses more extensively the growth of KML murine melanoma cells. We propose that deglycosylation of ELP is related to structural features of the protein, namely, the removal of oligosaccharide fragments unmasks the biological activity.

EXPERIMENTAL

Extensin-like proteins were isolated from 2-day cotton sprouts by the literature method [9].

Deglycosylation of Cotton ELP. Lyophilized material (3 mg) was placed into a plastic test tube and treated with anhydrous methanol (36 μl) and HF:pyridine (540 μl). The HF:pyridine mixture was prepared by adding to anhdyrous HF (420 μl) anhydrous pyridine (180 μl). The indicated amount was added to the sample. Cold water (3 ml) was added after 90 min incubation at room temperature. The precipitate that formed was separated by centrifugation at 1000 rpm for 10 min. The supernatant containing deglycosylated protein was dialyzed for 24 h against 0.1 M NaCl and 2 days against distilled water. The solid was treated with cold water and lyophilized. The procedure was repeated several times. The protein contents in the supernatant and precipitate were determined by the Lowry method; total sugars, by the anthrone—H_2SO_4 method.

KML cells (~80,000) were innoculated into a vial in nutrient medium (DMEM, 2 ml) with 10% bovine embryo serum, glutamine (200 mM), and antibiotics. They were cultivated in a CO_2-incubator at 37°C. ELP and d-ELP were added after 24 h in doses of 100 μg/ml. ^{3}H-Thymidine was added stepwise after the cells and medium were contacted for 24 h. Further treatment of the cells, incorporation of ^{3}H-thymidine, and counting of live cells were carried out according to the literature [13].

Gossypol and other toxic phenolic compounds were determined as follows. The sample (1 mg) was treated with a 10-fold excess of acetone. The mixture was ground in a mortar and centrifuged. The supernatant and solid were treated with phloroglucine (2%) in 2 M HCl. The color did not change in both fractions (a reddish color develops in a positive reaction).

The protein content was determined by the Lowry method [21].

The quantitative content of total sugars was determined spectrophotometrically using anthrone—H_2SO_4, as described in the literature [9].

All experiments, with the exception of the control, were repeated three times. The control was cells that were not treated with the preparation.

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