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Localisation of the protein containing hydroxyproline in the cells of *Dictyostelium discoideum*, a cellular slime mould

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ABSTRACT

Three methods were used to prepare plasma membranes of the cellular slime mould *Dictyostelium discoideum*. The concentration of combined hydroxyproline was determined in vegetative cells.

Hydroxyproline was found in the plasma membrane of both glucose and no-glucose grown cells in the form of protein containing hydroxyproline. This type of protein remains unchanged during differentiation and can be detected in fruiting bodies, and its localisation is in the plasma membrane.

INTRODUCTION

For many years there has been agreement that the primary cell wall of plants is a network of cellulose microfibrils. The discovery of a novel hydroxyproline-rich component in the cell wall has led to speculation that wall polysaccharides might be cross-linked via this protein to form a network in which covalent linkages were cleaved in order to allow cell extension (Lamport 1964, 1967).

Initial support for this hypothesis came from the tenacity with which the protein resisted attempts at removal from the cell wall. Enzymic degradation of cell walls released hydroxyproline-rich glycopeptides which also contained arabinose and smaller amounts of galactose. Analysis of these glycopeptides showed that the arabinose was *o*-glycosidically linked to hydroxyproline (Lamport 1969) and also that the glycopeptide contained at least one unit of pentapeptide, Ser-(Hyp) 4. These hydroxyproline-rich glycopeptides are probably derived from the protein 'extensin' which is also rich in hydroxyproline (White *et al.* 1973) and constitutes some 5% of the dry weight of the cell wall.

Hydroxyproline has also been found in the protein of the cell walls of algae (Thompson & Preston 1967), and many members of Phycomycetes have hydroxyproline in the protein of their cell walls too. This observation was confirmed by Novaes-Ledieu *et al.* (1967) and suggests that the protein may resemble that observed in wall preparations from algae and higher plants.

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The absence of a free hydroxyproline pool in plant cells and the fact that exogenous hydroxyproline was not incorporated into protein led to the suggestion that hydroxyproline arises from peptidyl proline (Pollard & Steward 1959) as in the better known case of animal collagen (Ramachandran 1967). The polypeptide backbone is synthesised on polyribosomes (Sadava & Chrispeels 1973) afterwards. Certain proline residues are hydroxylated via a soluble peptidyl proline hydroxylase (Lamport 1964). Following proline hydroxylation, a second series of post-translational modifications occurs in the protein, that of glycosylation. The initial point of attachment of any *o*-glycosidic bond from the 4-hydroxyl of the amino acid is to the 1-carbon of pentose sugar (Lamport 1967). Up to three more arabinose residues may be attached to the initial one. A particular UDP-arabinosyl transferase catalyses arabinose addition to hydroxyproline residues (Sadava & Chrispeels 1973).

During the next 7–10 min a completed glycoprotein is secreted to the cell wall (Miller & Lamport 1972). Secretion is an independent event and does not depend upon the continuation of the events preceding it. And it is an energy-requiring process which is mediated by membranes.

Although *Dictyostelium discoideum* is a member of the Myxomycota which are not generally regarded as being closely related to green plants, it is nevertheless thought that the mechanism of cell wall synthesis in this organism may throw some light on the same process in plant cells.

One of the principal attractions of *D. discoideum* is the separation of its life cycle into a unicellular stage during which individual amoebae are apparently indistinguishable from one another, and a multicellular stage during which a series of morphogenetic events involve cells with a clearly different functional characteristic and which are accompanied by extensive shifts in the metabolite content of the cells. These changes occur during 24 hr of differentiation when the myxamoebae stop dividing and differentiate into a fruiting body containing two cell types: stalk cells and spore cells. During development, major changes occur in cell metabolism. These changes include the synthesis of cell wall material in the fruiting body (Firtel & Bonner 1972).

The molecular organisation and the components of cell membranes in the vegetative myxamoebae and the cell wall of the stalk and the spores have been extensively studied. These studies have revealed that the cell membranes of the vegetative myxamoebae were found to be composed of 65–70% of dry weight of protein and 25–30% of lipids. Chemical assays (using an amino acid analyser) did not detect the presence of hydroxyproline (Yanagida & Noda 1969).

In *D. discoideum*, 95% of the hydroxyproline was found to be located in the vacuole of the vegetative cells which leaked out from the cells during differentiation. Less than 5% of the hydroxyproline was in the hydrolysed protein of the vegetative cells (Younis *et al.* 1980). It has been shown that this hydroxyproline-containing protein was not degraded during differentiation since its concentration remains unchanged (Younis *et al.* 1979, 1980).

MATERIALS AND METHODS

Growth and differentiation of the slime mould

D. discoideum cells of strain Ax-2 were grown axenically as described by Watts & Ashworth (1970). Cells were washed and allowed to differentiate as described by Younis *et al.* (1979).

Isolation of cell membranes

Three separate procedures were used:

(1) The procedure of Rossomando & Cutler (1975). Stationary phase cells were harvested from liquid culture by centrifugation at 5,000 ***g*** for 5 min and the pellet resuspended in TNT buffer (0·1 M Tris-HCl, pH 7·5; 0·1 M NaF; 0·02% DTT) at a final concentration of $1{\cdot}3 \times 10^8$ cells/cm^3. Amphotericin B (Fungizone) was added at a final concentration of 360 μg/cm^3 (by dilution from a stock solution of 5 mg/cm^3) and the cells incubated with shaking for 60 min at 22°C. After this incubation, light microscopic examination showed that 99% of the cells had lysed. Unlysed cells were removed from the lysate by centrifugation at 5,000 ***g*** for 5 min in a Sorval RC-2B centrifuge (p-5000). The resulting pellet was washed twice with TNT buffer to remove entrapped membrane material and the supernatant from each wash combined with the original 5000 ***g*** supernatant (s-5). Membrane structures were recovered from combined supernatants by centrifugation at 30,000 ***g*** for 30 min (p-30). The supernatants (s-30) were carefully and completely removed with a capillary pipette and the 30,000 ***g*** pellet containing the membrane structures removed. This pellet was washed once in TNT buffer and resuspended in 1 cm^3 of the same buffer for subsequent purification.

A final purification was accomplished by density gradient centrifugation through a discontinuous sucrose gradient composed of densities in g/cm^3 of 1·17 (39·9% w/v), 1·18 (41·6%), and 1·19 (42·6%). To form the gradient, 9 cm^3 of a solution of each density were layered into a 40 cm^3 polyallomer centrifuge tube. The sample was prepared by adding an equal volume of the 1·17 g/cm^3 sucrose solution to the resuspended p-30 pellet. The entire sample, about 2–3 cm^3, was layered on top of the gradient and the fractionation completed by ultra-centrifugation at 100,000 ***g*** for 18 hr (MSE Superspeed 65 Ultracentrifuge). The interphase layers were removed from the top with a capillary pipette, diluted 1:10 with 0·1 M Tris-HCl, pH 7·5 and recovered from solution by centrifugation at 30,000 ***g*** for 30 min. The pellets resulting from this centrifugation were resuspended in 10 cm^3 of the same buffer and washed twice by centrifugation to remove sucrose. All procedures were carried out at 4°C.

(2) The procedure of Yanagida & Noda (1969). Washed myxamoebae were mixed rapidly with 25 times their volume of 2·4 M sucrose in 0·1 M KCl-0·01 M phosphate buffer at pH 7·0 at 0°C. About 250 cm^3 of the suspension in a 500 cm^3 conical flask were kept at 3°C with constant stirring (200–300 rpm). Two days later, aggregates of the cell membranes appeared near the surface of the solution. These aggregates of membranes were separated by using 4 layers of nylon muslin, washed with a solution consisting of 0·12 M KCl and 0·01 M phosphate buffer at pH 7·0 and then rapidly washed with chilled water three times by centrifugation at low speed. The final sediment was hydrolysed directly and the hydroxyproline content assayed.

(3) The procedure of Green & Newell (1974). Washed myxamoebae were suspended in cold 10% (w/v) sucrose – 10 mM N-tris (hydroxymethyl) methylglycine-NaOH buffer, pH 7·5 (sucrose-tricine buffer) at a concentration of 1×10^9 cells/cm^3. Portions (5 cm^3) in plastic centrifuge tubes were frozen rapidly in liquid N_2 for 5 min and then allowed to thaw at room temperature until the homogenate temperature reached 4°C. The homogenate was immediately centrifuged at 700 ***g*** for 20 min in swing-out rotor of

refrigerated Sorvall Superspeed RC2-B Centrifuge. The pellet (cell membranes) was resuspended in sucrose-tricine buffer and homogenised with five up-and-down strokes. The homogenate was then made up to 50% (w/v) in sucrose and overlaid with sucrose zones of decreasing density. Centrifugation of the sucrose density gradient tubes was carried out at 4°C. Fractions were collected from the gradient by using Pasteur pipettes with bent tips. The hydroxyproline content was assayed after hydrolysis.

Hydroxyproline assay

Samples of the cell membranes were hydrolysed and the hydroxyproline was determined using the method of Blumenkrantz & Asboe-Hansen (1973). In samples containing low concentrations of hydroxyproline (less than 3·6 $\mu g/10^8$ cells, for which the optical density was less than 0·1 nm), concentrated samples at a cell density of 1×10^9 cells/cm^3 were taken and the results were corrected to 1×10^8 cells/cm^3.

Separation of spore and stalk cells

Differentiated cell samples of glucose and no-glucose grown cells were adjusted to 1×10^8 cells/cm^3. Stalk cells were separated from spores by using four layers of nylon muslin. Each sample was sonicated for a total of 2 min using 20-sec bursts. Aliquots of 2·1 cm^3 of 11·4 N HCl were added to each 2 cm^3 sample to make a final molarity of 6N HCl. Samples were allowed to stand for 1 hr to precipitate the protein, then centrifuged for 15 min in glass tubes at 3,000 rpm at 4°C. The resulting pellets were dried and weighed. The residues were hydrolysed with 6N HCl and dried in a vacuum desiccator at 60–70°C overnight.

RESULTS AND DISCUSSION

Of the three methods described in the literature for preparing plasma membranes (Yanagida & Noda 1969; Green & Newell 1974; Rossomando & Cutler 1975) two were mechanical methods of rupturing the cell and one (Rossomando & Cutler 1975) was chemical, using the antibiotic Fungizone. All three samples of membrane contained hydroxyproline, but the yield and purity of the membranes prepared using Fungizone were much better, for reasons discussed by Rossomando & Cutler (1975). Therefore, only the results of hydroxyproline concentrations in cell membranes and plasma membranes which were isolated by the method of Rossomando & Cutler (1975) are presented and discussed.

To detect the sensitivity of the method to separate free hydroxyproline and combined hydroxyproline (such as protein containing hydroxyproline), the concentration of free hydroxyproline (Table 1) was determined without hydrolysis. Total (free plus combined) hydroxyproline was determined after hydrolysis (Table 2). Comparison of these two tables shows that free hydroxyproline is quite absent from the purified plasma membrane fraction which only contains hydroxyproline as a component of glycoprotein or protein. As can also be seen in Table 1, there is no appreciable difference in the hydroxyproline content of plasma membranes of glucose and no-glucose grown cells.

The secretion of carbohydrates has been studied in the slime mould, *D. discoideum*, especially in relation to cell wall formation (Raper & Fennell 1952; George & Hohl 1972). However, little is known about the mechanism whereby this secretion occurs

Table 1. The concentration of hydroxyproline in unhydrolysed crude membrane and pure plasma membrane in $\mu g/10^8$ cells of myxamoebae (method of Rossomando & Cutler 1975)

Expt	Glucose grown cells (5,000 *g*)	Glucose grown cells (30,000 *g*)	No-glucose grown cells (5,000 *g*)	No-glucose grown cells (30,000 *g*)	Glucose and No-glucose grown cells (10,000 *g*)
1	0·92	0·03	0·72	0·01	0
2	0·50	0·10	0·81	0·02	0
3	0·89	0·02	0·63	0·05	0
4	0·62	0·01	0·80	0·07	0
Mean	0·73	0·04	0·74	0·04	0

Table 2. The concentration of hydroxyproline in hydrolysed crude membrane and plasma membrane in $\mu g/10^8$ cells of myxamoebae (method of Rossomando & Culter 1975)

	Glucose grown cells			No-glucose grown cells		
	Crude membrane		Plasma membrane	Crude membrane		Plasma membrane
Expt	(5,000 *g*)	(30,000 *g*)	(100,000 *g*)	(5,000 *g*)	(30,000 *g*)	(100,000 *g*)
1	2·00	1·16	0·61	1·40	1·53	0·42
2	0·98	0·81	0·86	1·72	0·87	0·62
3	1·30	1·94	0·93	0·76	1·20	0·87
4	0·95	1·42	0·52	0·87	1·47	0·84
Mean	1·30	1·33	0·73	1·18	1·26	0·68

and, in particular, whether it is associated with glycoprotein of the 'extensin' type. We have found no evidence for the synthesis, during differentiation, of any hydroxyproline-containing protein of the cell membrane which plays a role in cell wall synthesis. Previous studies (Yanagida & Noda 1969) on the protein components of the vegetative myxamoebae cell membranes revealed that protein was the major component of the cell membranes (65–70% of the dry weight is protein). Because of the low sensitivity of the amino acid analyser to hydroxyproline and the low content of hydroxyproline in the cell membranes (0·9 $\mu g/10^8$ cells, Younis *et al.* 1980), and in view of the results of Yanagida & Noda (1969), we expected to find almost no hydroxyproline among the amino acids detected by the amino acid analyser.

The specific method for hydroxyproline detection revealed that hydroxyproline is present in the cell membranes of the vegetative myxamoebae (Table 1). Since the expected amount of hydroxyproline to be found in the protein of the vegetative myxamoebae is at a maximum of 0·9 $\mu g/10^8$ cells (Younis *et al.* 1980), the concentration of hydroxyproline found in plasma membranes (0·73 $\mu g/10^8$ and 0·68 $\mu g/10^8$ cells) indicates that most of the hydroxyproline is present in the vacuoles and the smaller part

is in the protein whilst the localisation of proteinaceous hydroxyproline was in the plasma membranes.

In no-glucose grown cells and glucose grown cells, the concentration of hydroxyproline in the protein of the vegetative myxamoebae and differentiated cells was found to be 0·88 $\mu g/10^8$ cells (Younis *et al.* 1980). Thus, the data for proteinaceous plasma membrane (0·73 $\mu g/10^8$ and 0·68 $\mu g/10^8$ cells for glucose grown and no-glucose grown cells respectively), appear to be reasonable. Therefore, the localisation of the protein containing hydroxyproline of the vegetative myxamoebae is only in the plasma membranes of the cells. The slight differences of the cell membrane hydroxyproline of glucose and no-glucose grown cells is unappreciable (since the accuracy of the reading is up to $\pm 0{\cdot}05$ μg).

Changes in structure of myxamoebae occur during the formation of the fruiting body which consists of stalk cells and spore cells, so that the myxamoebae develop into stalk cells or spore cells. In the stalk cells of both glucose and no-glucose grown cells, the hydroxyproline was 0·33 $\mu g/10^8$ cells and 0·057 $\mu g/10^8$ cells respectively (Table 3).

Table 3. Hydroxyproline content of spore and stalk cells ($\mu g/10^8$ cells) of glucose (G) and no-glucose (NG) grown cells harvested at the fruiting body stage

Growth medium	Stalk dry wt. (μg)	Stalk hyp. (μg)	Stalk hyp. (% wt.)	Spore dry wt. (μg)	Spore hyp. (μg)	Spore hyp. (% wt.)	Total dry wt. spore + stalk (μg)	Total hyp. spore + stalk (μg)
NG	4,810	0·047	98×10^{-5}	75	1·181	1·575		
NG	8,125	0·068	84×10^{-5}	150	0·500	0·333		
Mean	6,467	0·057	88×10^{-5}	112	0·840	0·750	6,579	0·897
G	5,900	0·290	49×10^{-4}	160	0·072	0·045		
G	4,200	0·380	90×10^{-4}	240	0·123	0·051		
Mean	5,050	0·335	66×10^{-4}	200	0·195	0·097	5,250	0·535

Although there is no published work on the presence of protein in the stalk cell walls, the results of Table 3 indicate the presence of hydroxyproline in the stalk cell walls.

The spore wall is a multilayered structure containing both protein and cellulose (Loomis 1975). The outer layer contains galactose, so that this layer is a mucopolysaccharide. The middle layer has two distinct cellulose layers which differ only in orientation of their fibrils. The innermost layer contains cellulose and protein (Hohl & Jehli 1973). Since the innermost layer is the only remaining layer of the wall after spore germination and represents the future cell membranes of the vegetative myxamoebae (Yanagida & Noda 1969), and the hydroxyproline was detected in the cell membranes of the vegetative cells (Younis *et al.* 1980), therefore the presence of hydroxyproline in the spore cell walls (Table 3) was expected. It is most likely that the protein containing hydroxyproline is present in the innermost layer of the spore cell wall which undergoes no change during spore germination.

Although there is a slight difference in the amount of hydroxyproline in fruiting bodies derived from glucose and no-glucose grown cells, this difference seems unlikely to be appreciable since it is not certain that all of the cell walls were isolated. However,

it is clear that the hydroxyproline is present in the protein and that this protein is found to be localised in the cell membranes of the vegetative cells and in the cell walls of the stalk and the spores. Accordingly, a new question arises and that is whether many other micro-organisms have protein containing hydroxyproline.

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موقع البروتين الحاوي للهيدروكسي برولين في خلايا الفطر الهلامي ديكتيوستيليوم ديسكويديوم

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خلاصة

استعملت ثلاث طرق في تحضير الأغشية البلازمية للفطر الهلامي ديكتيوستيليوم ديسكويديوم ، وحسبت تركيزات الهيدروكسي برولين المتحد في الخلايا الخضرية . وقد ثبت وجود الهيدروكسي برولين في الأغشية البلازمية للخلايا النامية في وجود الجلوكوز وفي عدم وجوده ، وذلك على هيئة بروتين محتو على الهيدروكسي برولين . ان هذا النوع من البروتين يظل ثابتا ولا يتغير خلال مراحل التميز ، ويمكن الكشف عنه في الجسم الثمري ، حيث يتواجد في الأغشية البلازمية .

* العنوان الحالي : المعهد الطبي الفني ، باب المعظم ، بغداد ، العراق .
** العنوان الحالي : قسم وقاية النبات بكلية الزراعة ، أبو غريب ، بغداد ، العراق .