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RELATIONSHIP BETWEEN EVOLUTIONARY AGE AND HYDROXYPROLINE-CONTAINING MACROMOLECULES IN UNICELLULAR ALGAE

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Abstract—For 11 species of unicellular marine algae there is an inverse relationship between evolutionary age and hydroxyproline content of the high MW fraction of the cells. The increase in hydroxyproline content with decreasing evolutionary age is paralleled by a decrease in serine content. For an Antarctic clone of the diatom *Stauroneis amphioxys*, the level of hydroxyproline extracted from cells grown at 2° was four times that from cells grown at 16°. The distribution of hydroxyproline between the cell wall and cytoplasmic fractions varied with the state of growth of the cultures. In cells harvested during log phase, the highest concentration of hydroxyproline was in the cytoplasm, whereas in cells harvested at stationary phase, hydroxyproline was equally distributed between the wall and cytoplasmic fractions.

INTRODUCTION

The cell walls of higher plants and the extracellular matrix of animals both contain high MW glycoproteins characterized by their content of the imino acid hydroxyproline [1,2]. This fact, and the observation that the tertiary structure of these two glycoproteins (extensin in higher plants, collagen in animals) is highly conserved [1], supports Aaronson's [3] suggestion that the major matrix glycoproteins of both plants and animals have a common ancestry. In this paper we present evidence for an inverse relationship between the amount of hydroxyproline in high MW preparations from unicellular algae and their evolutionary age.

RESULTS

Relationship between evolutionary age and hydroxyproline content of unicellular algae

There is an inverse correlation between the amount of hydroxyproline present in the high MW fraction of alkaline extracts of the cells, and their evolutionary age based on the fossil record (Tables 1 and 2; Fig. 1). The blue-green alga *Synechococcus* sp. and the dinoflagellate *Gymnodinium* sp. had no detectable hydroxyproline. The oldest species having detectable hydroxyproline is the haptophyte *Cricosphaera carterae*. The highest content was found in *Isochrysis galbana*, the most recent species in evolutionary terms. *Tetraselmis chuii* has a relatively high hydroxyproline content; it is believed to be relatively recently evolved, but the dating is not unequivocally established, as the fossil record of the Prasinocladales is incomplete [4].

Paralleling the decrease in hydroxyproline with evolutionary age, there was an increase in the levels of serine detected. The inverse relationship between hydroxyproline and serine is reflected in the ratios hydroxyproline-serine (Table 2). None of the other amino acids showed significant variation. For example, there was less than 1% variation in the levels of alanine detected in all samples. Full amino acid analyses of three of the algae examined are shown in Table 3. The total hydroxy amino acids and the sum of hydroxy amino acids, proline, glycine and alanine, is also shown in Table 2. This latter group of amino acids accounts for at least one-third of the total protein extracted in each case.

Proline was present even in the oldest cell types examined, *Synechococcus* sp. and *Gymnodinium* sp. Like the other amino acids, the levels of proline in all species examined were comparable, so that the hydroxyproline–proline ratio increased with decreasing evolutionary age (Tables 2 and 3).

The Antarctic diatoms secrete a prolific extracellular mucilage that is predominantly polysaccharide in composition but which contains a small protein component (*ca* 10% by weight) (unpublished observation). Analysis of the high MW mucilage revealed no

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Table 1. Taxonomic orders and evolutionary age (from fossil record) of species analysed

Species	Class*	Order*	Earliest consistent fossil record ($\times 10^6$ years)†
Synechococcus sp.	Cyanophyceae	Chroococcales	1900
Cricosphaera carterae	Haptophyceae	Prymnesiales	300
Gymnodinium sp.	Dinophyceae	Gynmodiniales	207§
Skeletonema costatum	Bacillariophyceae	Centrales	185
Nitzschia seriata‡	Bacillariophyceae	Pennales	70
Nitzschia sp.‡	Bacillariophyceae	Pennales	70
Stauroneis amphioxys	Bacillariophyceae	Pennales	70
Dunaliella tertiolecta	Chlorophyceae	Volvocales	60
Isochrysis galbana	Haptophyceae	Isochrysidales	0.01
Tetraselmis chuii	Prasinophycace	Parsinocladales‖	?‖

*From ref. [20].
†From ref. [17].
‡Unialgal but not bacteria-free.
§Assumes age equal to the Peridiniales.
‖Provisional taxonomic assignment.
¶Incomplete fossil record.

detectable hydroxyproline. Similarly, hydroxyproline was not detected in the growth medium of the diatoms.

Relationship between temperature of culture and hydroxyproline content of unicellular algae

The level of hydroxyproline measured varied with the temperature at which the algae were cultured. For *Stauroneis amphioxys*, about three times as much hydroxyproline was detected in extracts from cells grown at 2° as from the same clone grown at 16° (Table 4). A similar relationship was found for two species of *Nitzschia*; in this case, the content of hydroxyproline in cells grown at 4° was nine times that of cells grown at 16° (Table 4).

Relationship between stage of growth and hydroxyproline content of Stauroneis amphioxys

Analysis of the high MW material present in alkaline extracts of *S. amphioxys* showed variation of hydroxyproline content with stage of growth as well as with temperature (Table 5). In early log phase, the amount of hydroxyproline in the extracts was the same for cells grown at both 2° and 16°; the distribution of this hydroxyproline between the cytoplasmic and cell wall fractions was the same for both temperatures, with about twice as much being present in the cytoplasm as the cell wall fraction. As the cells approached stationary phase, there was a marked difference in both amount and distribution of hydroxyproline in the fractions from cells grown at different temperatures. Thus, for either temperature, approximately equal amounts of hydroxyproline were found in the two fractions. However, there was about four times as much hydroxyproline present in extracts of stationary phase cells grown at 2° as at 16°.

DISCUSSION

The speculation that there are phylogenetic analogies between the cell wall hydroxyproline-containing glycoproteins of plants (extensin) and animals (collagen)[3] is supported by analytical data showing that the blue-green algae and the red algae are devoid of hydroxyproline, while more recently evolved algae, higher plants and animals contain significant levels of this imino acid[1, 5]. These findings are consistent with the hypothesis that before an oxygen atmosphere became established, cells (such as the blue-green algae) contained proline, which was not hydroxylated to hydroxyproline. After an oxygen atmosphere evolved, the capacity for hydroxylation developed and hydroxyproline appeared. That proline, but not hydroxyproline, was present in two of the older species examined (*Synechococcus* sp. and *Gymnodinium* sp.) is consistent with the suggestion[1] that the codon assignments were established well before availability of oxygen and proline hydroxylase enabled post-translational modification of proline to hydroxyproline.

The question of why, with the availability of proline hydroxylase and the substrates proline and oxygen, the level of hydroxyproline continued to increase rather than reaching a stable level coinciding with maximum oxygen levels, cannot be answered on the basis of the data presented. One possible explanation is that there is a sequence of amino acids which signals hydroxylation points which evolved more slowly. However, other evidence[6] suggests that such a sequence may not be essential to hydroxylation. Another consideration is that photosynthetic organisms create their own oxygen supply, and their capacity for hydroxylation might therefore not be expected to be limited by atmospheric levels.

The precise function of hydroxyproline in the cell wall is not known, although there is some evidence

PHYTO 21:9 - F

Table 2. Amino acid analysis of high molecular weight fraction of alkaline extract from unicellular algae

Species	Hyp (mol% total amino acids)	Ser (mol% total amino acids)	Hyp–Ser (mol%)	Hyp–Pro (mol%)	Hyp + Ser + Thr (mol% total amino acids)	Ala + Pro + Hyp + Ser + Thr + Gly (mol% total amino acids)	Protein extract (g protein/ 100 g dry wt)
Synechococcus sp.	0	14.97	0	0	22.3	43.7	3.2
Cricosphaera carterae	0.03	14.86	0.2	1.4	22.3	42.6	4.4
Gymnodinium sp.	0.00	9.83	0	0	14.1	40.5	2.8
Skeletonema costatum	0.11	15.73	0.7	2.6	21.5	42.0	3.9
Nitzschia seriata (16°)	0.08	10.61	0.8	1.7	18.0	41.9	0.5
Stauroneis amphioxys (16°)	0.25	5.45	4.6	4.7	10.6	33.7	5.9
Tetraselmis chuii	0.28	7.82	3.6	5.2	15.4	41.8	3.9
Dunaliella tertiolecta	0.53	4.70	11.2	10.0	9.1	30.7	0.9
Isochrysis galbana	3.76	5.03	74.7	27.3	13.2	35.9	3.4

Table 3. Amino acid analysis of high molecular weight fraction of alkaline extract from three unicellular algae

Amino acid	Mol% of total amino acids			
	S. amphioxys (16°)	*S. amphioxys* (2°)	*Gymnodinium* sp.	*Isochrysis galbana*
Hyp	1.19	0.25	0	3.76
Asp	12.68	12.75	9.48	11.28
Thr	4.57	4.89	4.27	4.43
Ser	6.01	5.50	9.83	5.03
Glu	12.60	13.76	13.60	13.78
Pro	5.99	5.30	3.53	4.91
Gly	8.95	7.80	12.27	7.56
Ala	10.34	10.00	10.60	10.31
Val	6.76	6.90	6.07	7.02
Met	2.08	2.29	1.52	2.17
Ile	4.61	4.88	3.68	4.54
Leu	8.20	8.29	7.38	8.44
Tyr	1.85	2.27	1.74	2.36
Phe	3.80	3.71	2.80	3.58
Lys	5.98	6.38	7.30	4.72
His	0.77	1.00	1.91	1.17
Arg	3.25	3.81	3.78	3.71

Table 4. Relationship between temperature of culture and hydroxyproline content of unicellular algae

Species	Culture temp. (°)	Hyp (mol% total amino acids)	Ser (mol% total amino acids)	Pro (mol% total amino acids)	Protein extracted (g protein/ 100 g dry wt)
Stauroneis amphioxys	16	0.34	9.64	3.23	4.3
Stauroneis amphioxys	2	1.01	6.42	5.02	11.3
Nitzschia seriata	16	0.08	10.61	4.60	0.5
Nitzschia sp.	2	0.75	7.10	5.16	2.0

Table 5. Hydroxyproline present in alkaline extracts of cell wall and cytoplasmic fractions of *Stauroneis amphioxys* taken from different stages of growth from cultures maintained at 2° and 16°

Stage of growth	Culture temp. (°)	Hydroxyproline (mol% total amino acids)		μmol Hyp/ mg extract	Protein extracted (g protein/ 100g dry wt)
		Cytoplasmic fraction	Cell wall fraction		
Early log	2	1.04	0.53	1.6	2.0
Stationary	2	0.66	0.64	6.0	11.3
Early log	16	1.04	0.58	1.4	2.8
Late log	16	0.16	0.18	0.6	4.3

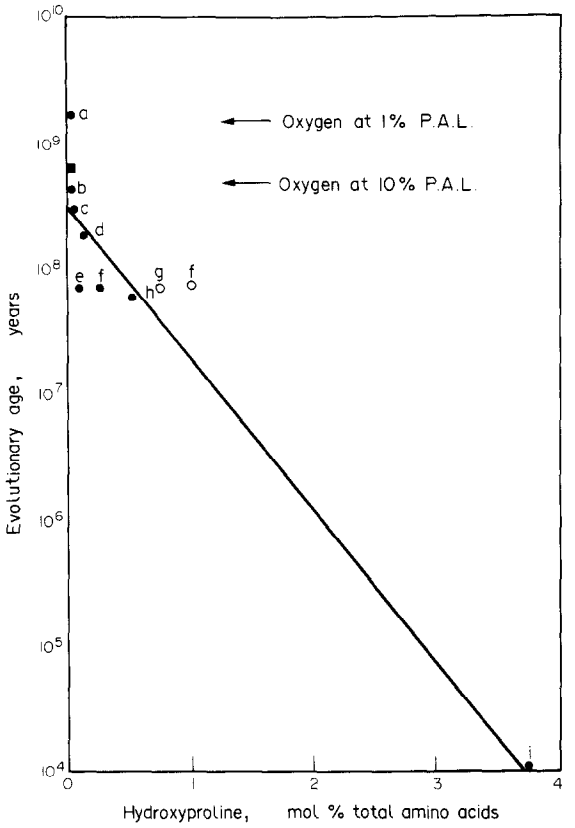

Fig. 1. Relationship between evolutionary age and hydroxyproline-containing macromolecules in unicellular algae. Evolutionary age was taken from the fossil record[17]; hydroxyproline content is expressed as mol% total amino acids in a high MW, alkali-soluble fraction of whole cells. The evolutionary times at which atmospheric oxygen was at 1% and 10% of the present atmospheric level (P.A.L.) are indicated (←) [18]. (●) Indicates data for unicellular algae cultured at 16°: (a) *Synechococcus* sp; (b) *Gymnodinium* sp; (c) *Cricosphaera carterae*; (d) *Skeletonema costatum*; (e) *Nitzschia seriata*; (f) *Stauroneis amphioxys*; (h) *Dunaliella tertiolecta*; (i) *Isochrysis galbana*; (○) indicates data for unicellular algae cultured at 2°: (g) *Nitzschia* sp.; (f) *Stauroneis amphioxys*; (■) indicates data for Rhodophyta taken from refs. [5] and [19].

that it fulfills a structural role, conferring rigidity to the wall[1, 2]. Another possibility is that the extracellular hydroxyproline-rich matrix was an essential component in the development of multi-cellularity, with individual cells being arranged precisely in the matrix developing from the primitive algal cell wall[1].

Our results demonstrate the relationship between evolutionary age and hydroxyproline content for a group of unicellular algae encompassing a broad evolutionary period from 1.9×10^9 years (*Synechococcus* sp.) to 1×10^4 years (*Isochrysis galbana*). The material examined was extracted from whole cells with alkali, by a procedure used for extracting hydroxyproline-containing glycoprotein from cell walls of beans[7]. This glycoprotein has only been extracted from higher plant cell wall fractions under degradative conditions. It is the major source of hydroxyproline in higher plant cells, although related macromolecules containing hydroxyproline are present in the cytoplasm and in plant cell secretions[8]. The metabolic relationships between these macromolecules are not defined[9]. The present study on the evolutionary age–hydroxyproline content relationship was on extracts prepared from whole cells (i.e. both wall and cytoplasmic fractions), which might contain a range of alkali-soluble proteins, glycoproteins, and other macromolecules, so that hydroxyproline and other amino acids measured would be derived from a number of different macromolecules. The extraction conditions used are efficient for higher plant cells and we assume a similar extraction efficiency for the algae examined in this study.

The inverse relationship between hydroxyproline and serine with evolutionary age is of particular interest in view of Aaronson's suggestion[3] that if hydroxy amino acids, in general, play an important role in structural proteins of plant cell walls and animal skeletons (through their capacity for glycosidic bond formation with carbohydrate), then they may accumulate over the evolutionary time span. For the unicellular algae studied, there is no increase in the group (Hyp, Ser, Thr) or the group (Ala, Pro, Ser, Hyp, Thr, Gly).

The finding that detectable levels of hydroxyproline were present in *C. carterae*, but not in *Gymnodinium* sp., may indicate that dinoflagellates like *Gymnodinium* sp. evolved prior to haptophytes. The age assignment quoted in Table 1 for *Gymnodinium* sp. is based on fossil data for the other major dinoflagellate group, the Peridiniales; however the primitive organization of the nucleus has led other authors to suggest that Gymnodiniales are organisms of great antiquity[10] and may indeed be older, in evolutionary terms, than *Cricosphaera*.

The increase in hydroxyproline in both *S. amphioxys* and two species of *Nitzschia* with decreasing temperature may be related to the increased solubility of oxygen at lower temperatures. A similar interpretation has also been proposed to account for control of lipid saturation in membrane preparations from the unicellular alga *Cyanidium caldarium*[11]. Alternatively, the hydroxyproline–temperature relationship may be a stress response similar to that observed in higher plants[12, 13].

The significance of the change of levels of hydroxyproline and their distribution between the cell wall and cytoplasmic fractions is not apparent. Since the major cell wall component of diatoms is silica, hydroxyproline is unlikely to be involved in controlling wall rigidity at least in diatoms, as has been suggested for higher plants[2]. However, 3,4-dihydroxyproline is present in the outer organic sheath of several diatoms[14] and this may be involved in controlling cell wall formation[15]. The question of the relationship of the 3,4-dihydroxyproline to 4-hydroxyproline and their possible role in wall formation is unresolved. In any case, it is apparent from these results that the growth stage and culture conditions should be considered in comparative studies of cell wall components.

EXPERIMENTAL

Algal species examined. A list of species examined is presented in Table 1; all cultures were uni-algal and clonal and were bacteria-free except where indicated. Cultures were handled aseptically. To produce cell material for analysis, rapidly dividing cells were taken from stock cultures to inoculate autoclaved f/2 medium[16] prepared from glass fiber-filtered Bass Strait sea-water. The cultures were maintained in 125 and 250 ml sterile flasks and were incubated without shaking at 17 ± 1° under cyclic illumination (14:10, light–dark) from Gro-Lux fluorescent lamps (110 μE/m^2/sec). The diatom *Stauroneis amphioxys*, isolated from pack ice in the Southern Ocean (67° 36′S., 60° 51′E.), was cultured at 2° and 16°.

When cultures reached late log-phase, cells were harvested by centrifugation (4000 *g*) for 10 min at 4°, and disrupted by sonication (two treatments each of 20 sec duration at 0.8 mA). The disrupted cell preparation was then centrifuged at 2300 *g* for 10 min, and the supernatant and residue were separately extracted with 2 M KOH at 20° for 24 hr under N_2[7]. The extract was filtered through a glass sinter (No. 1 porosity), the pH adjusted to 4.5 with HOAc and the neutralized extract was refiltered through a glass sinter (No. 4 porosity). The filtrate was dialysed against H_2O at 4°, concd, and freeze-dried. The amino acid content of the resulting preparation was determined after hydrolysis in 6 M HCl, 1% phenol for 24 hr at 110°, in a Beckman amino acid analyser. The amino acid composition of the growth medium which might contain secreted material was not examined.

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REFERENCES

1. Lamport, D. T. A. (1977) *Recent Adv. Phytochem.* **11**, 79.
2. Lamport, D. T. A. (1980) in *The Biochemistry of Plants* (Stumpf P. K. and Conn E. E., eds.) Vol. 3, p. 501. Academic Press, New York.
3. Aaronson, S. (1970 *Ann. N. Y. Acad. Sci.* **175**, 531.
4. Norris, R. E. (1980) in *Phytoflagellates*. Developments in Marine Biology Vol. 2. (Cox E. R., ed.) Elsevier–North Holland, Amsterdam.
5. Gotelli, I. B. and Cleland, R. (1968) *Am. J. Botany* **55**, 907.
6. Sadava, D. and Chrispeels, M. J. (1971) *Biochim. Biophys. Acta* **227**, 278.
7. Monro, J. A., Bailey, R. W. and Penny, D. (1975) *Carbohydr. Res.* **41**, 153.
8. Clarke, A. E., Anderson, R. L. and Stone, B. A. (1979) *Phytochemistry.* **18**, 521.
9. Chrispeels, M. J. (1976) *Annu. Rev. Plant Physiol.* **27**, 19.
10. Dodge, J. D. (1966) in *The Chromosomes of the Algae* (Godward M. B. E., ed.) pp. 96–115. Arnold, London.
11. Kleinschmidt, M. G. and McMahon, V. A. (1970) *Plant Physiol.* **46**, 290.
12. Adams, E. and Frank, L. (1980) *Annu. Rev. Biochem.* **49**, 1005.
13. Esquerre-Tugaye, M-T. and Lamport, D. T. A. (1979) *Plant Physiol.* **64**, 314.
14. Sadava, D. and Volcani, B. E. (1977) *Planta* **135**, 7.
15. Volcani, B. E. (1978) *Biochemistry of Silicon and Related Problems.* (Bendz G. and Lindqvist, T., eds.) 40th Nobel Symposium. Plenum Press, London.
16. Guillard, R. R. L. and Ryther, J. H. (1962) *Can. J. Microbiol.* **8**, 229.
17. Loeblich, A. R. (1974) *Taxon* **23**, 227.
18. Cloud, P. E. (1968) *Science* **160**, 729.
19. Schopf, J. W. (1970) *Biol. Rev.* **45**, 319.
20. Parke, M. and Dixon, P. S. (1976) *J. Mar. Biol. Assoc. U.K.* **56**, 527.