MATRIX-LYSIS AND RELEASE OF DAUGHTER SPHEROIDS IN *VOLVOX CARTERI* – A PROTEOLYTIC PROCESS

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Received 10 September 1979

1. Introduction

In the genus Volvocales we find the first simple organisms in which stem and somatic cells are differentiated and which establish an organized social hierarchy. Thus, they are interesting objects to study differentiation and ageing processes. In these multicellular green algae a number of systematic differentiating cell divisions and subsequent re-organization (inversion) of the cellular aggregate [1] produces:

(i) A sheath (which in some cases is syncytial) of numerous somatic cells which are fitted out with an eye-spot and two flagella for locomotion and protection. They excrete a matrix which consists of several outer layers of hydroxyproline-rich glycoprotein [2] and an inner gel, presumably a polysaccharide, degradable by lysozyme. Within this matrix are:

(ii) Vegetative gonidia in a fixed and limited number or, after a phase shift caused by the sexuality inducing glycoprotein [3,4], eggs and sperm-bundles. At maturation the vegetative daughter spheroids are released at a defined term, whereas the male spheroid disintegrates completely into sperm packets. For release each daughter spheroid dissolves the somatic sheath of the parental colony at a circumscribed area in their immediate neighbourhood and penetrates into the medium, possibly extruded by the elastic pressure of the matrix and starts to rotate vividly.

A lysis of cell walls is also described for zoospore liberation in *Chlamydomonas* [5]. In both cases an enzymatic reaction involving hydrolysis may be responsible. We were able to purify the enzyme from *Volvox carteri* to almost homogeneity and to characterize its catalytic properties as a neutral serine (glyco)protease.

2. Materials and methods

The female strain HK 10 and the male strain 60 1b (recl.) of *Volvox carteri* f. *nagariensis* Iyengar were obtained by courtesy of Dr R. C. Starr, Austin, TX. The algae were kept in soil water and grown axenically in Provasoli–*Volvox* medium [6] in a light (~9000 lux)/dark rhythm of 16:8 h at 28°C with sterile aeration. Under this regime the algae grow synchronously over several generations with a generation cycle of 48 h. For the production of larger amounts of cells we started with single colonies in 10 ml *Volvox* medium; after 3 generations the ~225 colonies were transferred to 200 ml medium and after 2 more generations (~25 000 colonies) from there to 800 ml medium, in which they were grown under further vigorous aeration for 6 days so that there were finally ~10^6 synchronous colonies, ready for release. The mature parental spheroids were let to settle and the bulk of the medium poured off. On letting the thick suspension stand without aeration in the dark for 1–2 h, the daughter spheroids hatch, and the autolysin is excreted into the medium from which it is isolated after removing the algae by filtration through 10 nm-mesh Nitex net.

The biological activity of solutions and preparations is measured by the following assay:

Mature parental spheroids are killed by adding 1% 35-proc. formaldehyde (or by heating a suspension

 Elsevier/North-Holland Biomedical Press

to 55°C for 10 min) and kept in the refrigerator*. Seven of the killed colonies (~100 daughter spheroids) are added to the samples (1 ml) to be assayed (or to appropriate dilutions in *Volvox* medium for quantification) and incubated in a 30°C water bath for 30 min. The liberated daughter spheroids are counted against a medium blank. Activity is given in % release.

For the assay of glycosidases the respective umbelliferyl glycosides were used as substrates, and the product measured fluorimetrically [7]. General protease activity was determined using casein as the substrate by ultraviolet spectrophotometry at 280 nm of the trichloroacetic acid supernatants [8]. The activity is expressed as TU^{Cas} = μmol tryptophan/ml (20 min, 30°C). Tryptic activity was followed by hydrolysis of *N*-benzoyl-L-arginine methyl ester; chymotryptic activity by using L-tyrosine ethyl ester [9]. Protein determination was by a modification of the Lowry procedure [10]. All chemicals were analytical grade.

3. Results

Release media liberate spheroids in the described bio-assay. They contain protease but no glycosidase activity. During release of the daughter spheroids the protein in the medium, the biological lysis activity and protease activity, all increase quickly and with a sigmoidal time dependency. However, as seen in fig.1, the lysis and the protease activity lag ~1–1.5 h behind the protein excretion.

Disintegration of mature colonies by sonication or by grinding with aluminum oxide show that lysis activity and protease activity increase within the cell at the same time and may be enriched from the homogenates. However, specific activities of such extracts are lower than that of the exoenzyme in the medium. Therefore the latter was used for further purification. The medium was concentrated and freed of the cells as described and lyophilized. The residues

* Heat-killed spheroids give ~35% lower activities than the formaldehyde-treated samples. In algae heated for 5 min to 55–56°C sufficient release activity is left to liberate their spheroids on standing. Very uniform preparations of spheroids and matrix may be obtained in that way for chemical analysis

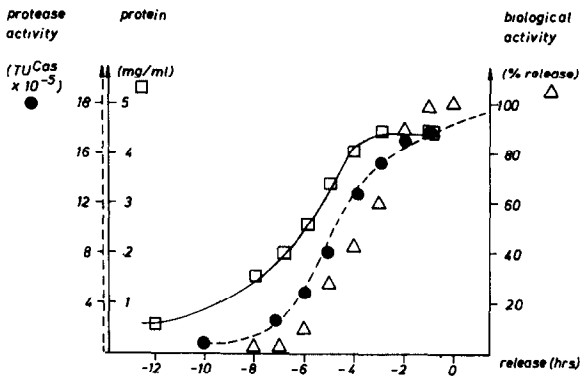

Fig.1. Protein content (□), Protease activity (●) and release activity (△) of medium during maturation of *Volvox carteri* spheroids. Time scale: hours before complete release of daughter spheroids.

from several runs were dissolved in water and protein precipitated with ammonium sulfate. The precipitate was spun down and redissolved in *Volvox* medium for further fractionation with ammonium sulfate. The lysis activity from non-induced HK 10 as well as from induced 69 1b was found in fractions at 45–55% saturation, the inducer glycoprotein precipitates at 65–75% saturation.

A further 10-fold purification was achieved by chromatography on Sephacryl 200-S. After this enrichment the biologically active fraction possesses solely protease but no glycosidase activity. The specific activity in the biological assay is ~25–40-times that of the medium. Purification with casein as substrate is given in table 1.

SDS gel electrophoresis (100 μg protein) of the active fraction shows a strong band at about 26 000 dalton and a faint one at higher molecular weight (fig.2). The lysis enzyme does not hydrolyze glucosides and galactosides; glucuronides, hyaluronides, pectin and sialyl glycosides. It is a neutral protease with a pronounced pH optimum of pH 7.4–7.6 (10^{-2} M imidazole). It is heat-labile with a half-time of 30 min at 50°C. The heat-inactivation kinetics is first order, pointing to only one active species of the lysis enzyme. The molecular weight is between 30 000 (Sephacryl) and 26 000 (SDS and disk electrophoresis). The enzyme is more acidic than the basic inducer glycoprotein and seems also to be glycosylated.

The hydrolase is stabilized by Ca^{2+}; phosphate

Table 1
Purification of *Volvox* matrix dissolving enzyme

	Vol. (ml)	Protein (mg)	Specific activity		*f*	Yield (%)
			Lysis (%)	Protease (TU^{Cas})		
Starting material (from 15 l lyophilized medium)	60	150	15	1.4×10^{-4}	1	100
Ammonium sulfate 45–55%	20	30	100	2.7×10^{-4}	2.7	54
Sephacryl (main fraction)	3	2.25	100	27×10^{-3}	27	41

(10^{-2} M) and EDTA (10^{-3} M) inhibit 60–80%; the activity can be restored by excess Ca^{2+} or Mg^{2+}. Heavy metal chelators (diethyl thiocarbamate, thioacetamide, α,α-dipyridyl, *o*-phenanthroline) do not inhibit. Inhibitors are: diisopropyl fluorophosphate (10^{-3} M = 100%), phenylmethyl fluorosulfate (10^{-3} M = 38%), benzamidine (10^{-4} M = 67%) diethyl pyrocarbonate (10^{-3} M = 100%), chloroquine (2×10^{-2} M = 45%), histidinol (10^{-3} M and 5×10^{-3} M = 38% and 76%, respectively) and *p*-chloro mercuribenzoate (10^{-3} M = 65%), whereas most other SH-blockers (5×10^{-3} M iodoacetamide) have no effect. Soybean trypsin inhibitor (0.15 mg and 1.5 mg/ml) blocks the enzyme 74% and 91% respectively, chymotrypsin inhibitors, pepstatin and kallikrein were without effect.

The lysis enzyme has limited species specificity. The sensitivity of different *Volvox* strains for the *V. carteri* release enzyme is as follows:

Euvolvox > *V. pocockiae* > *V. aureus* ≅ *V. carteri* HK 10 > *V. spermatoides* > *V. africanus* ≅ MET 1b.

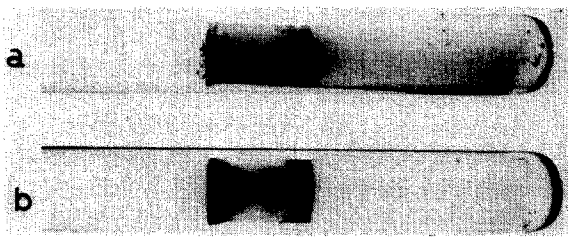

Fig.2. SDS–Gel electrophoresis of *Volvox*-matrix lysis: (a) 25 μg protein; (b) 100 μg protein.

Not lysed are the syncytial species *V. dissipatrix* and *V. gigas*. There is also no lysis of *Chlamydomonas reinhardtii*, and no cross lysis of *Volvox* by the *Chlamydomonas* autolysin (kindly provided by Dr U. G. Schlösser, Göttingen) which itself has a strong casein splitting activity (~80% of that of the *Volvox* autolysin) and which is also precipitated at 30–60% ammonium sulfate. The highly purified *Volvox* release enzyme is able to disintegrate killed but not living spheroids in vitro.

Daughter spheroid release and lysis of the matrix is blocked in vivo by: arginine (0.05 M), lysine (0.05 M), benzamidine (2.5×10^{-4} M) and by lectins, particularly concanavalin A, which at 3 μg/ml agglutinates the colonies. On the other hand, myristyl lecithin (15 μg/ml) causes premature liberation of the spheroids.

4. Discussion

The liberation of the daughter spheroids is effected by an enzymatic process, by which the sheath of the parental colony is pierced and lysed locally so that each daughter spheroid leaves through its own-made opening. The residual hull swims for a while actively in the medium and finally disintegrates. The lysis enzyme excreted into the medium is a slightly basic (glyco)protein with tryptic properties and serine and histidine residues in the active site. It seems to be excreted in form of a zymogen or pro-enzyme, and its activation, or des-inhibition, is time-dependent. We do not know whether the protease is formed in

the somatic cells or in the gonidia and whether it works from without or from within. It seems most plausible that the zymogen is activated during excretion, thus digesting the sheath from the inside. But the pro-enzyme could also be excreted first into the medium and activated during contact between parental somatic sheath and the daughter spheroid's flagellar border by an additional enzymatic system. This could also be the reason why the bio-assay works with killed spheroids and activates freshly added prohydrolase. Although autodigestion of healthy spheroids does not occur in the medium, injured spheroids are slowly decomposed by autolysin preparations, either because they are unable to protect themselves by an inhibitor of the autolysin or by binding of activators (such as Ca^{2+}). The regulation of this process may involve a cascade of destroying inhibitor(s), thus activating a protease which in turn activates the final autolysin, such as is the case with intracellular proteases [11]. The greater stability of heat-killed colonies compared with formaldehyde-killed colonies may point to such a mechanism, assuming that one of the intermediate reactions is impaired.

Acknowledgement

L. J. thanks Dr R. C. Starr for his hospitality during a sabbatical stay at Austin in summer 1977, when most of the present results were obtained.

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