Isolation and partial characterization of gamone 1 of *Euplotes octocarinatus*

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A polypeptide, termed gamone 1, was isolated and purified to homogeneity from culture filtrates of mating type VII of the freshwater ciliate *Euplotes octocarinatus*. The gamone induces intraclonal conjugation in cells of certain other mating types. The isolation and purification of the gamone was carried out by a combination of two chromatographic steps. The purified gamone was found to be still effective in a concentration of approx. 10^{-15}M.

Cell-cell interaction Euplotes octocarinatus *Conjugation induction* *Gamone*

1. INTRODUCTION

Ten mating types can be distinguished in the ciliated protozoon *Euplotes octocarinatus* at the present time. The mating types are determined by 4 codominant alleles (mt^1, mt^2, mt^3, mt^4) which control the production of 4 mating-inducing signal substances or gamones (G_1, G_2, G_3, G_4). They are secreted autonomously into the culture medium. These signal substances induce cells of certain other mating types to unite and to form conjugant pairs [1]. We are presently investigating the initial steps in preconjugant interaction and report here the isolation and partial characterization of gamone 1.

2. MATERIALS AND METHODS

2.1. *Culture conditions*

E. octocarinatus strain 1 (17)-VII (homozygous for the mating type allele mt^1) was grown in glass basins with permanent aeration in 20 l SMB-medium (according to [2], EDTA omitted) at 20°C. The cultures were grown on the green alga *Chlorogonium elongatum*, which in turn was cultivated in 5-l Erlenmeyer flasks in SMC (SMB + 1.25 mM NH_4NO_3, 0.009 mM $FeCl_3$, 0.5 μM $MnCl_2$) at 20°C with aeration in an 11 h light/13 h dark cycle.

To increase the secretion of gamone 1, cells of logarithmically growing cultures were concentrated by a recirculating mode filtration with a Pellicon cassette system (0.5 μm microporous filter) and resuspended in SMB at a density of 1×10^4 cells/ml. After 24 h at 26°C a cell-free fluid containing the secreted gamone was obtained by filtration as indicated above. The cells were resuspended once more in SMB, incubated for another 24 h and separated again from the culture fluid as described.

2.2. *Bioassay of gamone activity*

Evaluation of gamone activity in the cell-free fluid was performed using tester cells of strain R_7 (a mutant which appears not to secrete gamone but reacts with all known mating types) grown at 20°C in Fernbach flasks. A sample containing gamone activity was serially diluted with SMB by factors of 10 or 2. 250 μl of the diluted sample and 250 μl of a suspension of tester cells (containing approx. 1000 cells) were mixed and incubated for 5 h at 26°C. One unit of gamone activity (U) is defined

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as the smallest amount that induces the formation of at least 1 pair in a test volume of 500 μl and is standardized by comparison to a gamone 1 standard (cell-free fluid) stored at −30°C until use.

2.3. *Chromatographic media*

Hydroxyapatite (BRL), phosphocellulose P 11 (Whatman), DEAE-Sephadex A-25, CM-Sepharose G-25 and SP-Sephadex G-25 (Pharmacia) were treated as recommended by the suppliers. Gel filtration was performed using either a conventional chromatographic procedure with Sephacryl S-200 (Pharmacia) or an FPLC system with Superose 12 (Pharmacia).

2.4. *Analysis of proteins*

Samples were analyzed by electrophoresis on 10–20% polyacrylamide gradient gels [3] in the presence of SDS (fixed with methanol-acetic acid and stained with Coomassie brilliant blue R 250) or under nondenaturing conditions (fixed with perchloric acid and stained with Coomassie brilliant blue G 250, according to [4] but using 400 mg/l Coomassie). M_r standards were obtained from Sigma. Protein concentrations were measured according to [5] using bovine serum albumin as a standard.

3. RESULTS

Although cells of *E. octocarinatus* secrete gamones autonomously into the surrounding medium, the amount of gamone activity that is measurable in the cell-free fluid can be increased by incubating the cells at an elevated temperature (26°C) in an inorganic starvation buffer (SMB) for 2 × 24 h. In total, 10^8–10^9 U gamone activity can be obtained routinely from 2 × 10^8 cells suspended in 20 l SMB.

The gamone was found to be extremely stable. The cell-free fluid can be stored for months at 4°C without any significant loss of activity. Even incubation at 100°C for 5 min did not show an effect. After 15 min incubation at 100°C, 20% of the initial activity was still measured. Incubation of cell-free fluid with 50 μg/ml proteinase K for 30 min at 20°C led to a complete inactivation of the gamone.

To isolate gamone 1 from cell-free fluid, the binding of the gamone to different ion exchangers was analyzed. No adsorption to phosphocellulose, CM-Sepharose and SP-Sephadex was detected. The gamone bound to the anion exchanger DEAE-Sephadex with very low efficiency. When the gamone-containing fluid was applied to hydroxyapatite, gamone activity was adsorbed nearly quantitatively. Therefore gamone 1 was concentrated and purified by stirring 20 l of cell-free fluid (containing approx. 10^3–10^4 U/ml of gamone activity and < 0.01 μg/ml protein) with 0.2 g/l hydroxyapatite (dry wt; preequilibrated in SMB) for 30 min at 4°C. By this treatment more than 98% of the gamone activity of the batch bound to hydroxyapatite. Hydroxyapatite was allowed to settle down overnight, poured into a column (5 × 2 cm) and eluted with 0.2 M sodium phosphate buffer, pH 7.0 (fig.1). In the eluate (24 ml, < 1 μg/ml protein) approx. 10^5–10^6 U/ml of gamone activity could be measured. The low protein concentration of the hydroxyapatite eluate and a A_{280}/A_{260} ratio of 0.85 indicate that not all of the A_{280} monitored was due to proteins. Recombining the hydroxyapatite eluate with different fractions of the purification protocol did not show

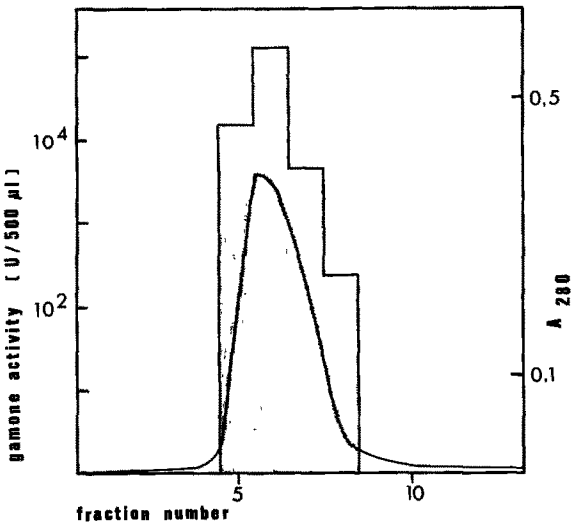

Fig.1. Hydroxyapatite elution profile. Gamone from 20 l cell-free fluid was bound to hydroxyapatite and eluted with 0.2 M sodium phosphate buffer, pH 7.0. 6-ml fractions were collected. (—) A_{280}. Gamone activity is indicated by the histogram.

any indication of chromatographic separation of an inhibitory or stimulatory compound present in the cell-free fluid, although this possibility cannot be excluded completely.

Fractions of the hydroxyapatite eluate containing gamone activity were pooled, dialyzed against deionised water overnight, lyophilized and subsequently dissolved in 1 ml of 50 mM Tris-HCl, pH 7.5, 0.1 M KCl. After centrifugation at 10000 × *g* for 10 min, the supernatant was applied in 200-μl aliquots comprising 3.9 μg protein to a gel filtration column performed by FPLC on Superose 12. As can be seen in fig.2, the gamone was eluted in a well-separated, single peak containing 2 μg protein and 10^4 U of activity per aliquot. By this step the gamone activity is significantly reduced. Recombining the different fractions of gel filtration, in particular those containing low-M_r components, was not found to restore the activity originally applied. In addition, recombination of the gamone-containing fractions derived after gel filtration with cell-free fluid or the hydroxyapatite eluate did not show any stimulatory or inhibitory effect.

After chromatography on Superose 12, the protein peak exhibiting the gamone activity was fur-

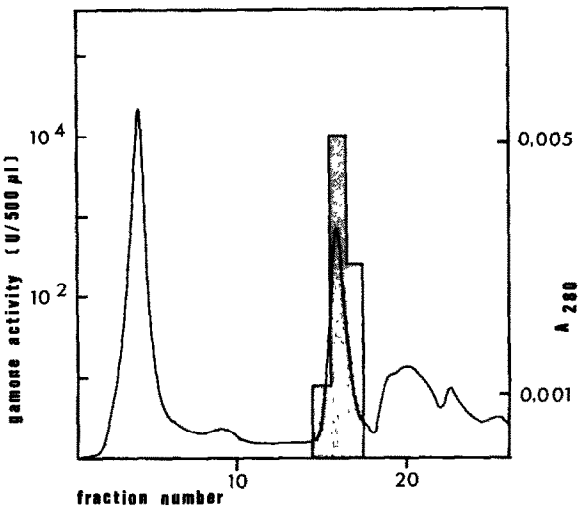

Fig.2. Superose 12 elution profile. 200 μl of the lyophilized hydroxyapatite eluate derived from 20 l cell-free fluid were applied to a Superose 12 column and eluted with 50 mM Tris-HCl, pH 7.5, 0.1 M KCl. 0.5-ml fractions were collected. (—) A_{280}. Gamone activity is indicated by the histogram

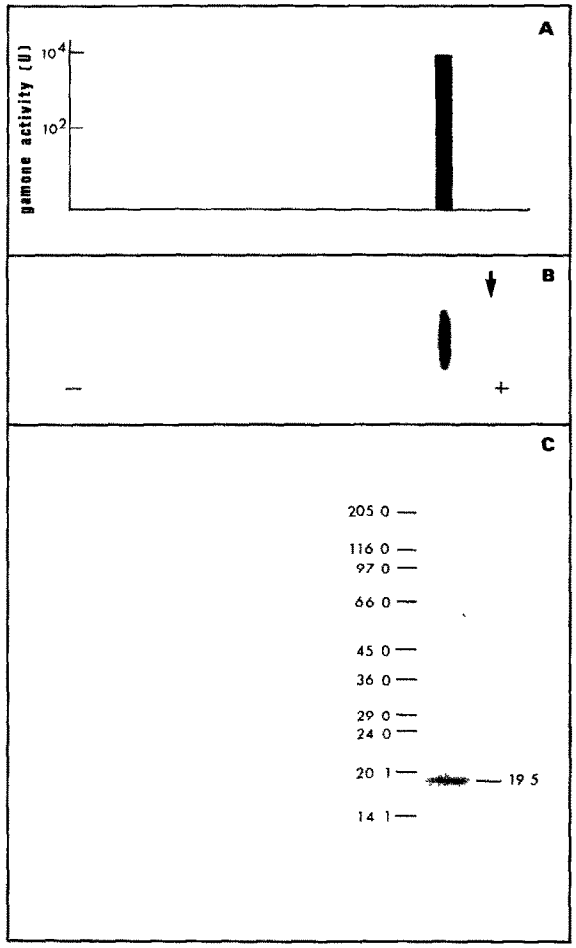

Fig.3. Polyacrylamide gel electrophoresis of gamone 1. Gamone from 50 l cell-free fluid was purified as described. The gamone-containing peaks derived from gel filtration were pooled, precipitated with 0.5 M trichloroacetic acid, divided into 2 identical aliquots and subjected to nondenaturing gel electrophoresis in a 10–20% polyacrylamide gradient gel in 2 parallel lanes (12.5 μg protein/lane). After electrophoresis one lane was stained with Coomassie brilliant blue G in perchloric acid [4] (B; arrow indicates the migration front), and the other cut into slices (0.5 cm). The gel slices were eluted by rigorous agitation in SMB for 24 h at 4°C and gamone activity in the eluates was determined (A). The stained polypeptide band shown in B was cut out and subjected to re-electrophoresis on a 10–20% SDS-polyacrylamide gradient gel. After staining with Coomassie brilliant blue R in a solution of methanol-acetic acid [3], a single band was obtained corresponding to an M_r of 19500 (C).

ther analyzed by polyacrylamide gel electrophoresis. Under nondenaturing conditions only a single protein band was obtained that contained solely the gamone activity (fig.3A,B). Re-electrophoresis of this protein band in the presence of SDS again showed only one band with an apparent M_r of 19500 (fig.3C). Since the gamone band obtained after electrophoresis under nondenaturing conditions cannot be fixed in alcoholic solvents, fixation by perchloric acid was performed to avoid diffusion of the protein out of the gel. After electrophoresis in the presence of SDS the best results were obtained if fixation was carried out with a methanol-acetic acid solution.

By addition of pure gamone 1, cells which do not produce this gamone could be induced to form pairs. On the other hand, cells which produce this gamone did not respond.

The apparent M_r of gamone 1 determined by gel filtration differs significantly from the value of 19500 found by SDS-polyacrylamide gradient gel electrophoresis and depends on the type of gel employed in the experiment. Using identical M_r markers and identical running buffers, the M_r of gamone 1 has been determined to be 14000 by conventional chromatography on Sephacryl S-200 and 25000 by FPLC on Superose 12.

According to the protein content of the gamone peak revealed after gel filtration on Superose 12, we can calculate that approx. 5 μg pure gamone 1 is obtained from 10 l cell-free fluid using the isolation procedure described here.

4. DISCUSSION

The isolation and purification of gamone 1 of the ciliate *E. octocarinatus* were carried out by a combination of 2 chromatographic steps. As monitored by native and denaturing polyacrylamide gel electrophoresis, no other protein besides a single polypeptide exhibiting the gamone activity could be detected in the gamone preparation after gel filtration.

Combination of various fractions derived during the isolation procedure gave no indication of a chromatographic separation of additional factors influencing the biological activity of the gamone.

Differences in the determination of the molecular mass of gamone 1 were revealed after SDS gel electrophoresis (19.5 kDa) and after gel filtration on Sephacryl S-200 (14 kDa) or Superose 12 (25 kDa). Similar differences in the determination of the molecular mass have been found for blepharmone, the gamone 1 of *Blepharisma japonicum* [6] and for euplomone r13, a gamone of *E. raikovi* [7]. Both are glycoproteins of comparable size. Supposing a molecular mass of 19.5 kDa and assuming a nearly quantitative isolation, the concentration of the gamone in the cell-free fluid was determined to be 10^{-10}–10^{-11} M under the conditions reported here. The gamone was found to induce pair formation at a concentration as low as 10^{-15} M. This value is lower than that found in the ciliates *B. japonicum* [8] and *E. raikovi* [7]. However, the measurement of the biological activity of gamones by induction of pair formation is influenced to a large extent by the sensitivity of the tester cells. Consequently, a comparison of the efficiency of gamones isolated from different organisms is limited. Taking this into account we can still say that the concentration and activity of gamone 1 of *E. octocarinatus* are comparable to those of pheromones isolated from other eukaryotes [9].

According to physiological and genetic data [1] *E. octocarinatus* strain 1(17)-VII (mt^1mt^1) is expected to secrete only one type of gamone into the culture fluid. Indeed, the gamone activity has been found to be exclusively associated with one polypeptide. Preliminary efforts of our group to isolate the other 3 gamones of *E. octocarinatus* indicate that all gamones are polypeptides which are similar in size but show charge differences (unpublished).

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REFERENCES

[1] Heckmann, K. and Kuhlmann, H.-W. (1982) J. Protozool. 29, 525.
[2] Miyake, A. and Beyer, J. (1973) Exp. Cell Res. 76, 15–24.

[3] Mueller, K., Freiburg, M. and Hoyer-Feige, G. (1985) J. Biochem., in press.
[4] Sedmak, J.J. and Grossberg, S.E. (1977) Anal. Biochem. 79, 544–552.
[5] Spector, T. (1978) Anal. Biochem. 86, 142–146.
[6] Braun, V. and Miyake, A. (1975) FEBS Lett. 53, 131–134.
[7] Miceli, C., Concetti, A. and Luporini, P. (1983) Exp. Cell Res. 149, 593–598.
[8] Miyake, A. and Beyer, J. (1974) Science 185, 621–623.
[9] Birch, M.C. (1974) Pheromones, North-Holland, Amsterdam.