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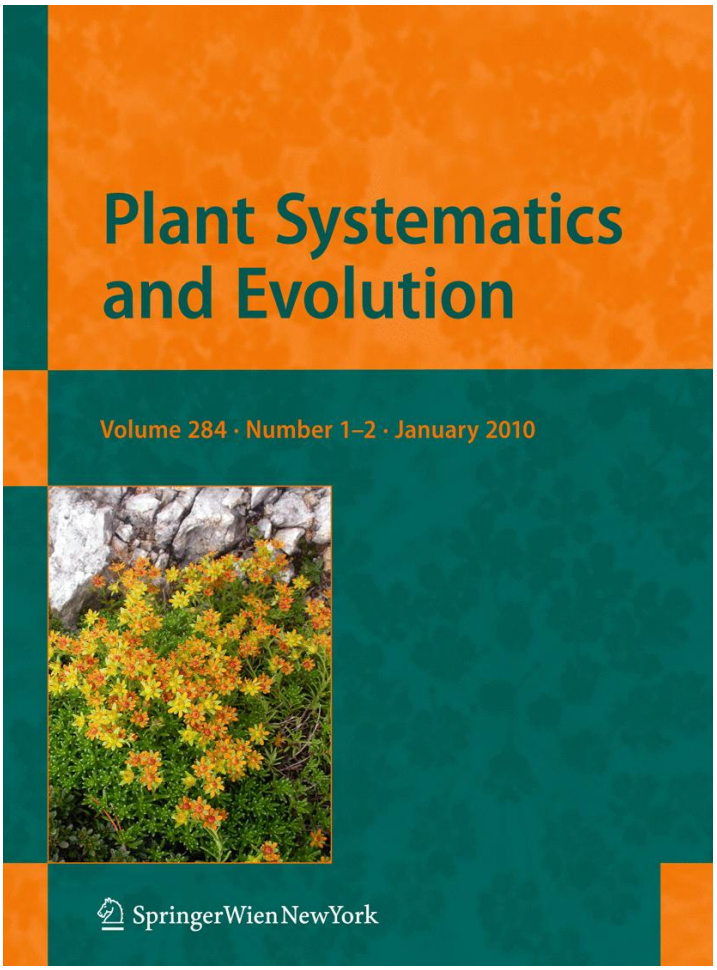

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ORIGINAL ARTICLE

Morphological features and isoenzyme characterization of endosymbiotic algae from green hydra

Goran Kovačević · Sandra Radić · Biserka Jelenčić · Mirjana Kalafatić · Hrvoje Posilović · Branka Pevalek-Kozlina

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Abstract Symbiotic associations are of a wide significance in evolution and biodiversity. Green hydra is a typical example of endosymbiosis. In its gastrodermal myoepithelial cells, it harbors individuals of unicellular green algae. Morphological characteristics of isolated algae determined by light and electron microscopy are presented. Cytological morphometric parameters (cell area, cell radius, chloroplast area) of isolated algae from green hydra (Cx), as well as from reference species *Chlorella kessleri* (Ck) and *Chlorella vulgaris* (Cv), revealed similarity between the isolated endosymbiont and *C. kessleri*. Isoenzyme patterns of esterase (EST), peroxidase (POX), and catalase (CAT) were used for the investigation of genetic variability in endosymbiotic algae isolated from green hydra. Out of 14 EST isoenzymes observed in Cx species, 9 were expressed in the Cx sample. Results of the EST isoenzyme analysis indicated a higher degree of similarity between Cx and Cv than between Cx and Ck. Due to much higher heterogeneity, EST isoenzymes seem to be more suitable genetic markers for identification of different *Chlorella* species than CAT and POX isoenzymes. Results obtained suggest that symbiogenesis in green hydra has probably not been terminated yet.

G. Kovačević (✉) · S. Radić · B. Jelenčić · M. Kalafatić · B. Pevalek-Kozlina
Division of Biology, Faculty of Science, University of Zagreb, Rooseveltov trg 6, 10000 Zagreb, Croatia
e-mail: goran@zg.biol.pmf.hr

H. Posilović
Department of Geology, Faculty of Science, University of Zagreb, Horvatovac 102a, 10000 Zagreb, Croatia

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Introduction

Hydra (Cnidaria, Hydrozoa) is an aquatic cosmopolitan invertebrate (Holstein and Emschermann 1995) that represents a very fine object for experimental purposes (Arkhipchuk et al. 2005; Beach and Pascoe 1998; Kalafatić and Kopjar 1995; Kovačević et al. 2001).

Green hydra (*Hydra viridissima* Pallas, 1766) is an endosymbiotic species that harbors individuals of unicellular green algae in its gastrodermal myoepithelial cells (Burnett 1973; Douglas and Smith 1984). In one cell up to 20 algae can be found (Dunn 1987; Holstein and Emschermann 1995) with each alga surrounded by a vacuolar membrane, forming a symbiosome (O'Brien 1982; Reisser and Wiessner 1984).

Individuals and species that do not establish an endosymbiotic relationship are called asymbiotic or nonsymbiotic, while organisms that can form endosymbiotic relationships in certain periods and then again live freely as separate organisms are called aposymbiotic organisms. The question is why some species can and others cannot establish the endosymbiotic relationship.

The capability of algae for symbiosis with green hydra correlates with the tolerance to low pH. A few hours after the infection, pH of perialgal vacuoles is 3.5–4 (Huss et al. 1993/1994). Symbiotic algae are acid-tolerant. Below pH 4, green algae constantly produce an increased amount of maltose (Kessler et al. 1991). Algae are mostly placed into a quiet part of the cell where no intensive intracellular decomposition occurs (McAuley and Smith 1982). Nonviable algae merge with the digestive vacuoles of

gastrodermal myoepithelial cells (O'Brien 1982). Endosymbiosis with one algal species established once precludes the same process with another species (Rahat 1991). Green hydra is the only hydra host of zoochlorellae.

It has been considered that endosymbiotic algae from green hydra belong to the *Chlorella* genus, a huge and complex genus of polyphyletic origin (Huss et al. 1993/1994). Algae of the *Chorella* genus are morphologically indistinguishable (Habetha et al. 2003). Particular species also belong to the class Chlorophyceae (Friedl 1997). About 350 genera and 10,000 species of green algae have been described (Mädgefrau and Ehrendorfer 1988). Species closely related to *Chlorella vulgaris* Beij. (K&H, 1992), such as *Chlorella kessleri* Fott et Novak. (K&H, 1992), belong to the class Trebouxiophyceae and only those represent the true genus *Chlorella*. Other species within *Chlorella* not so closely related to the mentioned species or those included into other developmental lines within Trebouxiophyceae and Chlorophyceae should be included in another genus (Friedl 1997).

Cytological morphometric analysis has been widely used as a scientific method in biology and biomedicine (Agostinucci et al. 2002; Baron et al. 2004; Tonar and Markos 2004), in taxonomy (Nakahara et al. 2003), and recently in symbiotic relationship research (Handa et al. 2006; Kaltenpoth et al. 2005; Kovačević et al. 2005).

One of the advantages of using isoenzymes to study polymorphism is that a range of enzyme loci on one individual can be studied easily using a small quantity of material with minimum preparation and cost.

Peroxidases (POX, EC 1.11.1.7) are ubiquitous enzymes found in virtually all green plants, many fungi, and aerobic bacteria. The isozymic heterogeneity of peroxidases appears to result from de novo synthesis, as well as an array of physiological/ecological determinants including hormones, light, gravity, and infection (Siegel 1993). Peroxidases have phylogenetically correlated similarities based on the chemical nature and redox potentials of the substrates they can oxidize. They catalyze the dehydrogenation of structurally diverse phenolic and endiolic substrates by H_2O_2 and are thus often regarded as antioxidant enzymes, protecting cells from the destructive influence of H_2O_2 and derived oxygen species (Vianello et al. 1997).

In many plants, the tetrameric heme-containing enzyme catalase (CAT, EC 1.11.1.6) forms cellular populations consisting of multiple forms (isoforms), which are usually located in peroxisomes. Catalase, which degrades H_2O_2 into water and oxygen, is one of the major antioxidant enzymes. Three CAT isoforms have been identified in maize on separate chromosomes; they are differentially expressed and independently regulated (Scandalios 1990).

Esterases (EST), a group of hydrolases, catalyze the formation or cleavage of ester bonds of water-soluble substrates. Generally, these enzymes have a broad spectrum of substrates and act on a variety of natural and xenobiotic compounds (Cummins et al. 2001). Naphthylacetates (1- and 2-) used for esterase activity and visualization in tested *Chlorella* species are substrates of arylesterases (EC 3.1.1.2) and of carboxylesterases (EC 3.1.1.1) (De Carvalho et al. 2003). The esterase zymograms contain a characteristic pattern of a large number of bands controlled by several loci (Cubadda and Quattrucci 1974). They have been used often to study intra- and interspecific polymorphism (Brown and Weir 1983).

Thus, the objective of the present study was to characterize the endosymbiotic algae isolated from green hydra based on morphological, cytological, and isoenzyme analysis and to compare them with the two reference free-living *Chlorella* species.

Materials and methods

In the experiments, the following test organisms were used: *Chlorella kessleri* Fott et Novak. (K&H, 1992) strain LARG/1 and *Chlorella vulgaris* Beij. (K&H, 1992) strain SAG 211-11b, and endosymbiotic algae isolated from green hydra.

Algae were maintained on the surface of a sterile deep stock agar according to Pratt (1941) and modified according to Horvatić et al. (2000).

Morphological and cytological characterization

Morphological and cytological characterization of endosymbiotic algae isolated from green hydra in comparison with aposymbiotic standard species *Chlorella vulgaris* and *Chlorella kessleri* was performed with binocular light microscopes (Reichert and Nikon Eclipse E600, programme Lucia G DXM1200 version 4.81, camera Nikon DXM1200). For further morphological analysis, fresh algae were fixed onto the holder and coated with carbon using sputter coater Bal-Tec SCD 050. The SEM micrographs were recorded using SEM Tescan TS 5136 MM in secondary electron mode at an accelerating voltage of 30 kV. The measured and compared parameters were cell area, cell radius, and chloroplast area. Measurements were performed on a sample of 200 cells for cell area and radius and 50 cells for chloroplast area. The experiment was performed in triplicate.

Enzyme extraction and soluble protein determination

Prior to homogenization, samples (150–330 mg) of *Chlorella kessleri* (Ck), *Chlorella vulgaris* (Cv), and endosymbiotic algae isolated from green hydra (Cx) were scraped off

the agar surface, collected in tubes, frozen in liquid nitrogen, and homogenized (TissueLyser, Qiagen) for 1 min at 30,000 Hz. Additional homogenization (10″) was performed following addition of 500 µl of ice cold 50 mM potassium phosphate (KPO_4) buffer, pH 7.0, containing 1 mM EDTA and 5 mM sodium ascorbate. The homogenates were then centrifuged at 3,000*g* for 10 min at 4°C (Sigma 3K18 centrifuge). Supernatants were used for enzyme activity and isoenzyme and soluble protein analysis. When determining the total amount of algae needed for isolation of soluble proteins, it was necessary to take a much smaller amount of endosymbiotic algae isolated from green hydra, almost one-third to one-half less than the other samples. Soluble protein content was determined by the method of Bradford (1976) using bovine serum albumin (BSA, Sigma) as standard.

Enzyme assays

The activity of nonspecific peroxidase was measured by monitoring the formation of purpurogallin at 430 nm ($\varepsilon = 2.47\ mM^{-1}\ cm^{-1}$) according to Chance and Maehly (1955). The reaction mixture contained 50 mM KPO_4 buffer (pH 7), 1 mM H_2O_2, 20 mM pyrogallol, and enzyme extract. Catalase activity was determined by the decomposition of H_2O_2 and was measured spectrophotometrically by following the decrease in absorbance at 240 nm (Aebi 1984). Activity was calculated using the extinction coefficient ($\varepsilon = 40\ mM^{-1}\ cm^{-1}$) and a unit of CAT was defined as micromoles of decomposed H_2O_2 per gram of dry weight (DW) per minute. Esterase activity was determined spectrophotometrically at room temperature (25°C) using either 1- or 2-naphthylacetate as substrate (Burlina and Galzigna 1972). Esterase activity was calculated following the increase in absorbance at 322 nm (for 1-naphthylacetate) or 313 nm (for 2-naphthylacetate), due to the formation of 1-naphtol ($\varepsilon_{322nm} = 2.0\ mM^{-1}\ cm^{-1}$) or 2-naphtol ($\varepsilon_{313nm} = 1.25\ mM^{-1}\ cm^{-1}$) (Balen et al. 2003). The reaction mixture contained 100 mM Tris/HCl, pH 7.4, and 100 mM 1- or 2- naphthylacetate, both dissolved in absolute methanol. The esterase activities were corrected for spontaneous hydrolysis of 1- and 2-naphthylacetate.

The specific enzyme activity for all enzymes was expressed as units per milligram of protein (1 U = µmol min^{-1}).

Statistical analysis

For each analysis, data were compared by analysis of variance (ANOVA), using STATISTICA 8.0 (StatSoft, USA) software package, and differences between corresponding controls and exposure treatment were considered as statistically significant at $p < 0.05$. Each data point is the average of three replicates unless stated otherwise.

Activity gel analysis

Tissue extracts were run with anodic gel electrophoresis under nondenaturating conditions using vertical 10% (EST, POD) or 7% (CAT) polyacrylamide slab gels with the buffer system of Laemmli (1970). A constant voltage of 200 V was applied for 4 h and the temperature maintained at 4°C. Equal amounts of protein were loaded onto each lane. For detection of POX isoforms, the gels were first soaked in 50 mM sodium phosphate buffer (pH 7.0) for 30 min and the bands visualized in the same buffer containing 4 mM H_2O_2 and 20 mM pyrogallol (Mittler and Zilinskas 1993).

Staining for CAT isoenzymes was carried out as described by Woodbury et al. (1971). Gels were washed in distilled water (3 × 15 min) and soaked in 5 mM H_2O_2 (10 min). After a brief rinse, the gels were incubated in 1% (w/v) ferric chloride/1% potassium ferricyanide solution and CAT isozymes appeared as colorless bands on a dark green field.

The esterase isozymes were visualized according to a modified procedure described by Balen et al. (2003). 1-Naphthyl acetate and 2-naphthyl acetate were dissolved in 50% (v/v) acetone and mixed with 50 mM Tris/HCl, pH 7.1. After staining (30 min), the gels were washed with tap water and incubated in a solution containing 50 mM Tris/HCl, pH 7.1, and Fast Blue RR salt until dark brown (1-naphthyl acetate) or purple red (2-naphthyl acetate) bands appeared (20–30 min). The Fast Blue RR salt was dissolved in absolute methanol and filtered into 50 mM Tris/HCl, pH 7.1. The gels were rinsed once more with tap water and fixed in 30% (v/v) ethanol.

Results

Qualitative characterization

Endosymbiotic algae isolated from green hydra were of coccoidal shape from the 1st to the 40th generation and of light green color. Cells divided into up to four autospores. From the 41st generation, algae started to grow in colonies and did not separate after division. Algae formed coenobia of up to four cells. Some of those showed empty cell lumens and were less viable or died. From the 43rd generation, algae completely changed their morphology from coccoidal to elliptical coenobial shape (Fig. 1). Cells in transition were also present. From the 46th generation, algae formed coenobia of up to seven cells. SEM confirmed the elliptical shape of the cells forming coenobia (Fig. 2).

In addition to their different shades of green color, endosymbiotic algae isolated from green hydra appeared to

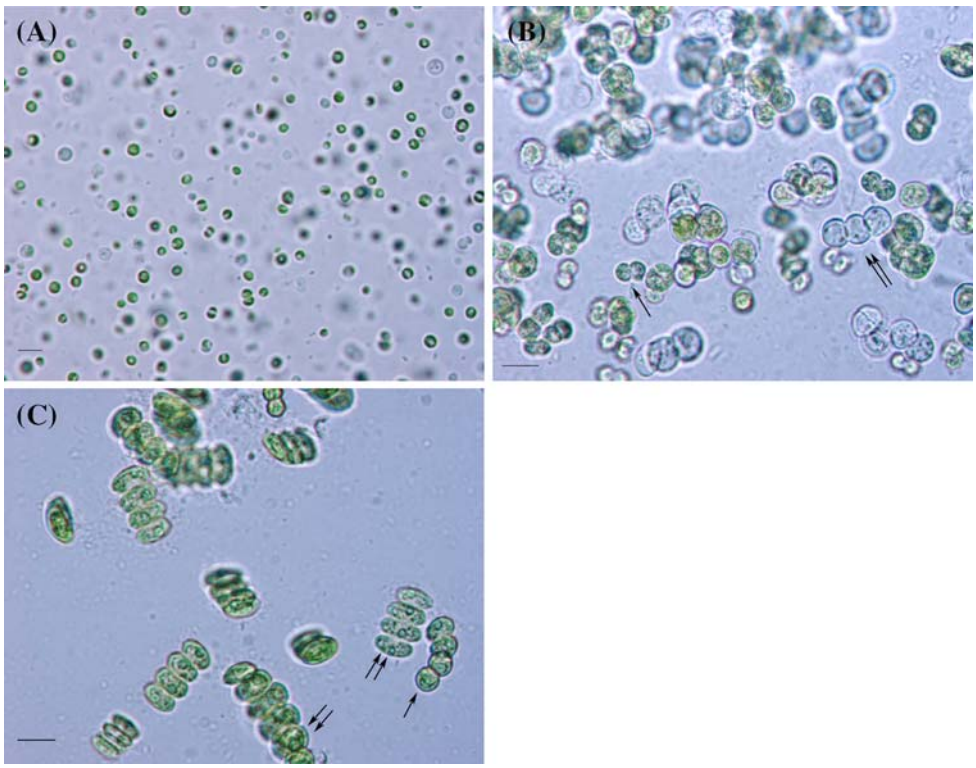

Fig. 1 Endosymbiotic alga isolated from green hydra. **a** 10–15 days after isolation. **b** 41st generation, the beginning of coenobia growth (*arrows*). **c** 43rd generation, coenobial growth (*arrow*) and changed algal morphology from coccoidal to elliptical form (*2 arrows*). *Bar* 5 μm

be noticeably more dry and of more granular structure than the other samples, enabling their easier handling and identification.

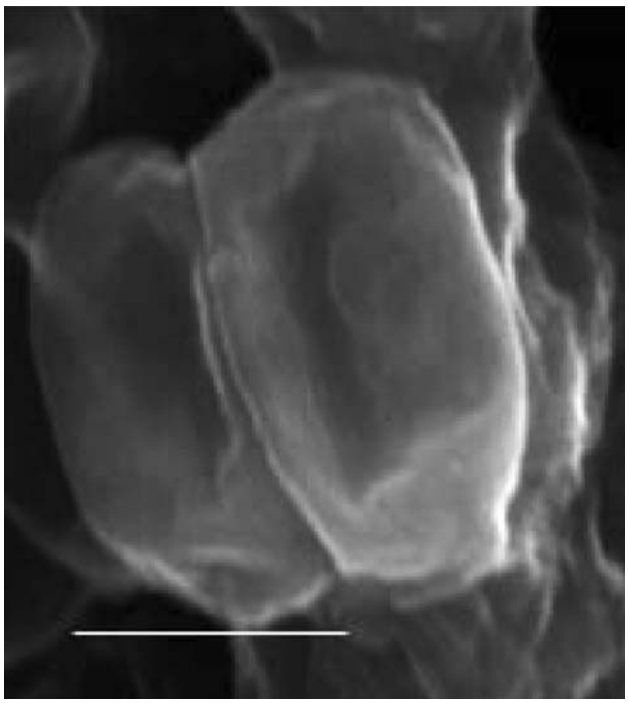

Fig. 2 SEM of endosymbiotic alga isolated from green hydra (43rd generation). *Bar* 5 μm

Morphometric characterization

Three morphometric parameters of tested *Chlorella* specimens were measured: cell area, cell radius, and chloroplast area (Table 1). *Chlorella kessleri* (Ck) and endosymbiotic algae isolated from green hydra (Cx) showed similar values for cell and chloroplast areas, whereas 50% smaller values of the parameters were found for *Chlorella vulgaris* (Cv). Regarding cell radius, values declined in the order Ck > Cx > Cv.

Peroxidase activity and isoenzyme analysis

The highest POX activity was observed in the Ck sample (Fig. 3a). Cx and Cv samples exhibited 34 and 63% lower activity, respectively, in comparison with the Ck sample. Native electrophoresis revealed five isoperoxidases in tested *Chlorella* samples (Fig. 3b). Four isoenzymes (POX1-4) were noted in the Ck sample, three (POX2, 4,

Table 1 Morphometric analysis of *Chlorella vulgaris* (Cv), *Chlorella kessleri* (Ck), and *Chlorella* isolated from green hydra (Cx)

	Cell area	Cell radius (μm)	Chloroplast area
Cv	6.31 ± 0.12	1.28 ± 0.014	2.98 ± 0.059
Ck	12 ± 0.27	2.24 ± 0.017	8.22 ± 0.144
Cx	12.95 ± 0.26	2.17 ± 0.030	8.83 ± 0.160

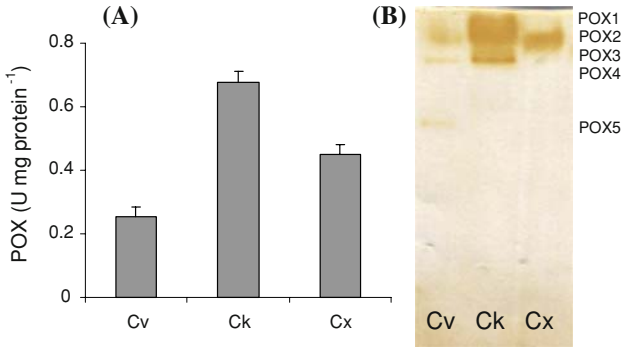

Fig. 3 Peroxidase (POD) activity (**a**) and isoenzyme analysis (**b**) in *Chlorella vulgaris* (Cv), *Chlorella kessleri* (Ck), and endosymbiotic *Chlorella* isolated from green hydra (Cx). Values are mean ± SD based on three replicates

and 5) in the Cv sample, while in the Cx sample only POX2 was expressed.

Catalase activity and isoenzyme analysis

As in the case of POX, CAT activity was highest in the Ck sample (Fig. 4a). Cv and Cx samples showed 56 and 79% lower activity, respectively, in comparison with the Ck sample. Four anodic CAT isoenzymes were observed in tested *Chlorella* samples (Fig. 4b), of which all four were present in the Ck sample, three (CAT1, 3, and 4) in the Cv sample, and only one (CAT1) in the Cx sample.

Esterase activity and isoenzyme analysis

Esterase activity was evaluated with two substrates, 1- and 2-naphthyl acetate. The enzyme activity of the Ck sample measured with 1-naphthylacetate was considerably higher than in the Cv sample, but the highest activity was found in the Cx sample (Fig. 5a). The esterase activity of tested

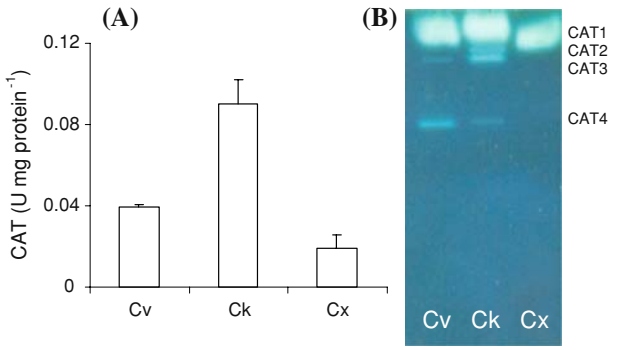

Fig. 4 Catalase (CAT) activity (**a**) and isoenzyme analysis (**b**) in *Chlorella vulgaris* (Cv), *Chlorella kessleri* (Ck), and the endosymbiotic *Chlorella* isolated from green hydra (Cx). Values are mean ± SD based on three replicates

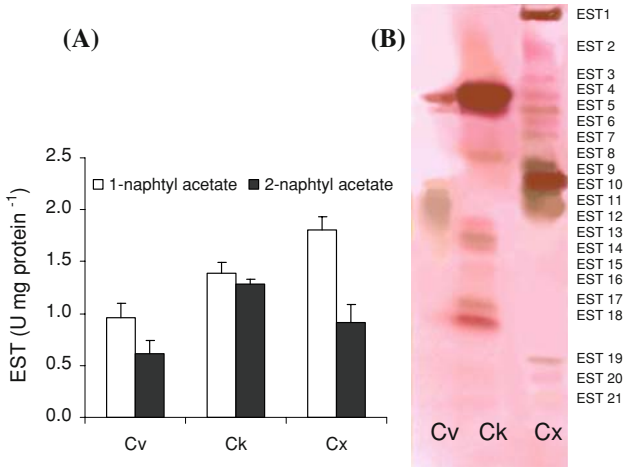

Fig. 5 Esterase (EST) activity (**a**) and isoenzyme analysis (**b**) in *Chlorella vulgaris* (Cv), *Chlorella kessleri* (Ck), and the endosymbiotic *Chlorella* isolated from green hydra (Cx). Values are mean ± SD based on three replicates

Chlorella species assayed with 2-naphthylacetate significantly declined in the order Ck > Cx > Cv.

Results presented in Fig. 5b show the variations in the esterase isoenyzme pattern of *Chlorella vulgaris* (Cv), *Chlorella kessleri* (Ck), and the endosymbiotic *Chlorella* isolated from green hydra (Cx). The esterases were numbered in sequence starting from the anode, according to their decrease in negative charge. In total, 21 esterase isoenzymes were resolved in three algal samples but only isoesterases EST4 and 5 were common to all samples. In addition to those, isoesterases EST10-12 were expressed in the Cv sample (five in total). Seven additional isoenzymes, EST8 and EST13-18, were expressed in the Ck sample only. Electrophoretic pattern of the Cx sample revealed 14 isoesterases in total: 9 isoesterases (EST1-3, EST6-7, EST9, EST19-21) not present in either of the referent *Chlorella* (Cv and Ck) samples, 3 isoesterases present in the Cv sample (EST10-12) but not in the Ck sample, and 2 isoesterases common to all tested algae (EST4-5).

The differential staining pattern showed that EST7, EST 9, EST11-12, EST14, and EST19 isoenzymes hydrolyze 1-naphthyl acetate; EST1-4, EST6, EST13, EST16, EST18, and EST20 isoenzymes hydrolyze 1-naphthyl acetate, and EST5, EST8, EST10, EST15, EST17, and EST21 isozymes hydrolyze both 1- and 2-naphthyl acetate.

Discussion

Morphometric characteristics are quantitative characteristics that have a complex genetic basis, with the environment exerting a strong influence on phenotypic variations within populations (Falconer 1986; Lewontin 1974). According to Fawley et al. (2004), unicellular green algae

display characteristics of environmental plasticity. If survival capacity is increased by a changed phenotype, morphological plasticity can provide a selective advantage (Diaz-Pulido et al. 2007).

In the case of green hydra symbiosis, only unicellular symbiosome formation is possible inside the gastrodermal myoepithelial cells. After their isolation, endosymbiotic algae became aposymbiotic, and their plasticity changes from coccoidal to elliptical form. Almost 3 years after the culture initiation, all isolated aposymbiotic algae displayed the elliptical shape. Isolated algae that could not follow the plasticity transformation into a coenobial form lost almost 100% of their viability. Hegewald and Hanagata (2000) showed that the coenobial ellipsoid *Dicloster acuatus*, formerly classified within the family Scenedesmaceae, is more related to *Chlorella kessleri*.

Phenotypic plasticity is estimated by the number of cells in one coenobium. It is not clear which mechanisms determine the number of cells per coenobium. Our isolated symbionts consisted of up to seven cells per coenobium, which probably represents the plasticity pattern needed for survival of the aposymbiotic system. Based on morphometric parameters, higher similarity was noticed between the isolated endosymbionts and *C. kessleri* than between the isolated endosymbionts and *C. vulgaris*.

In general, changes in the isoenzyme (CAT, POX, EST) patterns were consistent with the quantitatively measured enzyme activities in tested algae. Results obtained by the native anodic electrophoresis suggest complex relations within the *Chlorella* genus. The results of CAT and POX isoenzyme analysis indicate that the endosymbiotic *Chlorella* isolated from green hydra is equally related to both reference algae from *Chlorella* genus. However, based on the huge esterase polymorphism, a higher similarity was observed between the isolated endosymbiotic *Chlorella* and *Chlorella vulgaris* than between the former and *Chlorella kessleri*. In addition, nine EST isoenzymes were specifically expressed in the isolated endosymbiotic alga, which points out that they undoubtedly group in a different category.

Esterase isoforms specific to *C. kessleri* (seven isoforms) and endosymbiotic *Chlorella* (nine isoforms) may either arise *de novo* or due to posttranscriptional modification of the genes (Gershater and Edwards 2007). In contrast to the EST results, only limited heterogeneity (one to five isoforms) of CAT and POX was noticed in the tested *Chlorella* species.

Due to the high polymorphism of EST isoenzymes in the tested species, esterases could serve as useful genetic indicators for identification of different *Chlorella* species.

With long-term maintenance of cultures, it became obvious that endosymbiotic alga from green hydra were morphologically different from the reference species. Endosymbiotic alga displayed specific green coloring and appeared drier and more granular than the other samples and, as such, could easily be recognized among the samples. In addition, compared to the reference *Chlorella* species, much smaller quantities (almost one-third to one-half) of endosymbiotic algae were required for the soluble protein analysis. A lateral eukaryote/eukaryote gene transfer has recently been suggested between hydra and endosymbiotic algae (Habetha and Bosch 2005). It has also been noticed that some algae are placed very close to the host nucleus (Habetha et al. 2003). This position might have enabled mutual transfer of genetic material between hydra and endosymbiotic algae. A clearer insight into the phylogenetic status of hydras themselves, maintenance of symbiosis and course of symbiogenesis in green hydra is desirable.

In conclusion, symbiosis can significantly alter life on Earth. Symbiosis is not a side or a sporadic event. Symbiotic organisms are abundant, diverse, and significant. This significance is characterized by biodiversity, regulation, maintenance, and improvements of the symbiotic mechanisms in the present association. Symbiosis is an important biological principle whose recognition is unavoidable for understanding of the living world. Concerning the morphometric similarity of isolated algal endosymbionts with the free-living relative *C. kessleri* and isozyme similarity with the free-living relative *C. vulgaris*, it could be concluded that symbiogenesis in green hydras is an ongoing process.

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